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Investigating novel transglycanase activities within the plant kingdom

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DECLARATION

This thesis and the work of which it is a record was composed by myself. Where substantial input has been made by third parties, acknowledgement is made on the following page. This work has not been submitted for any degree or professional qualification other than that specified.

Claire Holland

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For Jamie,

with all my love,

and for Elisabeth and Colin Gould,

who I hope I've done proud.

Abstract

Integral to the physiological and biochemical properties of the plant, the primary cell wall (PCW) is of great economical interest. Transglycanases are a class of cell-wall remodelling enzymes hypothesised to be involved – among other functions – in cellular elongation and strengthening of the PCW. At present only four transglycanases have been convincingly characterised but the potential existence of many more is likely. To detect potential novel transglycanase activity, broad spectrum fluorescent and radioactive assays were conducted using a variety of potential donor and acceptor substrates. Enzyme extracts were sourced from a range of plants that represented the majority of the plant kingdom. Beansprout extracts reproducibly displayed significant incorportation of radioactivity and fluorescence when incubated with an α-arabinan or β-galactan donor and labelled xyloglucan oligosaccharide (XGO) acceptor. However, further analysis indicated the presence of xyloglucan contamination in donor polysaccharides and thus the activity observed was xyloglucan endotransglucosylase (XET). It has been hypothesised in the literature that linkages formed between the hemicellulosic and pectic matrices may be due to the activity of a transglycanase. This study has found no evidence to support this.

In addition, during identification of the gene responsible for mixed-linkage β-(1,3),(1,4)-glucan : xyloglucan endotransglycosylase (MXE) activity – observed in *Equisetum* – a heterologous *Pichia pastoris* expression system was developed allowing the synthesis of a novel recombinant hetero-transglycanase (HTG) conferring predominant MXE activity and of five previously unstudied recombinant XET-active xyloglucan *endo*transglycosylase/ hydrolases (XTHs).

Abbreviations

α-arabinan (αA) = α-(1→5)-Arabinan

AG = Arabinogalactan

Ara = Arabinose

Arx = Arabinoxylan

β-galactan (βG) = β-(1→4)-Galactan

BAW = Butanol/ acetic acid/ dH_2O

cpm = Counts per minute

dH_2O = Distilled water

EAW = Ethyl acetate/ acetic acid/ dH_2O

EPyAW = Ethyl acetate/ pyridine/ acetic acid/ dH_2O

Fuc = Fucose

Gal = Galactose

GalA = Galacturonic Acid

GAX = Glucuronoarabinoxylan

GlcA = Glucuronic Acid

Glm = Glucomannan

Glx = Glucuronoxylan

GGM = Glucuronoglucomannan

GH = Glycoside Hydrolase

HEC = Hydroxyethyl cellulose

HTG = Heterotransglucanase

IP = Isoprimeverose

Man = Mannose

Man_6 = β-(1→4)-Mannose hexasaccharide

MeGlcA = Methylated GlcA

MeOH = Methanol

MET = Mannan *endo*transglycosylase

MLG = Mixed linkage β-(1→3), (1→4)-glucan

MLGO = MLG oligosaccharide

MXE = MLG : xyloglucan *endo*transglucosylase

NRT = Non-reducing terminal

OAD = Oligosaccharidyl-1-amino-1-deoxyalditol

PCW = Primary cell wall

pMXE = Purified MXE extract from crude *Equisetum fluviatile*

PyAW = Pyridine/ acetic acid/ dH_2O

RAR = Reductive amination reagent

RG-I/ -II = Rhamnogalacturonan-I/ -II

Rha = Rhamnose

RT = Reducing-terminal

SR = Sulphorhodamine

TLC = Thin-layer Chromatograpy

TFA = Trifluoroacetic acid

Tx = Tamarind xyloglucan

WSCA = Water-soluble Cellulose Acetate

XB = Xylobiose

XEH = Xyloglucan *endo*hydrolase

XET = Xyloglucan *endo*transglucosylase

XGO = Xyloglucan oligosaccharide

XTH = Xyloglucan *endo*transglycosylase/ hydrolase

XXXG = Xyloglucan-derived heptasaccharide

[^{3}H]XXXGol = Reductively tritiated XXXGol

XyG = Xyloglucan

Xyl = Xylose

Xyl_6 = β-(1→4)-Xylose hexasaccharide

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1 Introduction

1.1 The primary plant cell wall

Functionally diverse and chemically unique from the extra-cellular material of other organisms, the plant cell wall has potential for high economical gain in the fibre, food and pharmaceutical industries. Many physiological characteristics of the cell – size, shape and function – are determined by the variable properties of its cell wall. The primary cell wall (PCW) encompasses an organised network of cellulose microfibrils tethered by hemicellulose cross-links integrated within a matrix of pectin, hemicelluloses and cell wall proteins. This structure has sufficient strength to resist turgor pressure whilst remaining dynamic, allowing remodelling of the cell wall which is central to cell elongation.

1.1.1 Cellulose

Cellulose is a long, linear homo-polysaccharide comprising β-(1→4)-D-glucopyranose residues (Figure 1) which is synthesised at the plasma membrane by large multi-meric cellulose synthase (CesA) complexes (Endler & Persson, 2010). Cellulose adopts an extended stiff-rod formation devoid of branching. Hydrogen-bonds formed between adjacent glucose residues on neighbouring cellulose chains results in the formation of highly rigid microfibrils. The scaffold formed by the association of these microfibrils provides resistance to the internal osmotic pressure of the cell as a result of its high tensile strength (O'Sullivan *et al*., 1997; Gorshkova *et al*., 2010). Cellulose is the predominant, and most abundant, load-bearing polysaccharide of the cell wall. Cellulose is insoluble in water and the majority of organic solvents, biodegradable and contains both crystalline and amorphous domains.

The orientation of cellulose microfibrils dictates cell shape and differs between the primary and secondary cell walls. Microfibrils are aligned parallel to each other along the fibre axis in the secondary cell wall to create an organised scaffold

while the microfibrils in the PCW are often less ordered, sometimes occupying all orientations (O'Sullivan *et al.*, 1997).

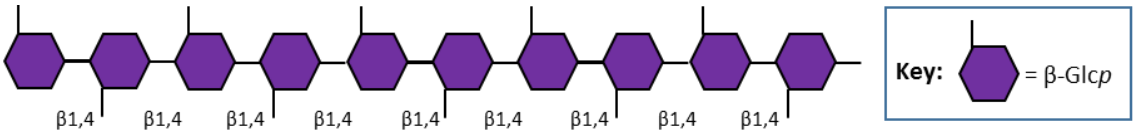


Figure 1: Cellulose structure

1.1.2 Hemicelluloses

Hemicelluloses are defined as a group of cell wall polysaccharides containing a β-(1→4)-linked backbones mainly synthesised by glycosyl transferases in the Golgi membranes (Scheller & Ulvskov, 2010). Despite their amorphous structure which provides little strength and confers susceptibility to enzymic attack they are functional as flexible cross-links between cellulose microfibrils and between pectic substances (Cosgrove, 2000; Zykwinska *et al.*, 2005, 2006). The main classes of hemicellulosic polysaccharides – xyloglucan (XyG), mixed-linkage glucan (MLG), xylans and mannans – are described below:

1.1.2.1 Xyloglucan

Xyloglucan is the best-characterised hemicellulose of the PCW; usually a neutral polysaccharide (Peña *et al.*, 2008) with a β-(1→4)-D-glucopyranose backbone substituted 50-75% with α-(1→6)-D-xylose side-chains (Scheible & Pauly, 2004). Side-chains are often capped with β-D-galactose (sometimes extended by α-L-fucose) or α-L-arabinose residues but a more extensive range of branching patterns has been observed in plant cell walls (Figure 2). XyG is ubiquitous in the embryophytes but has not been definitively observed in algal species, indicating a significant change in cell wall content during plant evolution, suggesting a pivotal role for XyG in adaption of species for the transition from colonisation of water to land (Popper & Fry 2003, 2004, 2008).

The pattern of backbone substitution can vary significantly between different plant tissues and species (Vincken *et al.*, 1997). In the majority of dicots, XyG is composed of repeating cellotetraose subunits of which the first three glucosyl residues (from the non-reducing terminal (NRT)) are substituted with α-(1→6)-D-Xyl side-chains; represented as XXXG in the nomenclature described in Fry *et al.* (1993). Other typical dicot XyG repeat units include XXGG (primarily in solanaceous plants) (Vincken *et al.*, 1997) XXLG, XLXG and XLFG (Johansson *et al.*, 2004). The predominant xyloglucan oligosaccharides (XGOs) produced via depolymerisation of tamarind seed XyG are XXXG, XXLG, XLXG and XLLG (Buckeridge *et al.*, 1992; Marry *et al.*, 2003). Poacean XyG, meanwhile, differs significantly from that of many other species with a variable subunit length of $XX(G)_n$ (where n=1-4) (Hsieh & Harris, 2009; Gibeaut *et al.*, 2005).

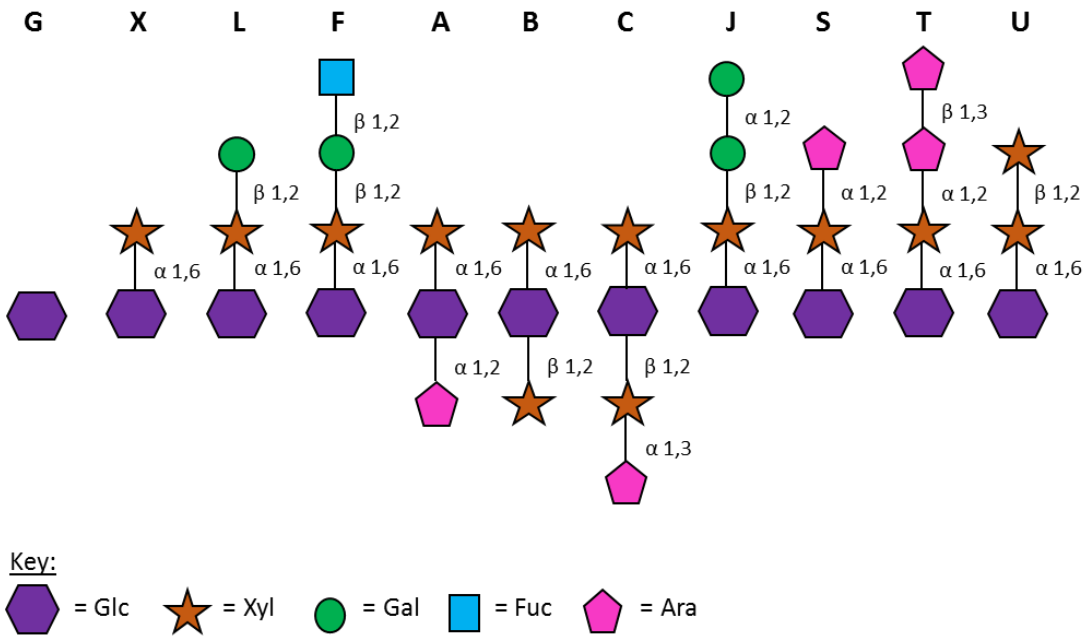


Figure 2: Common XyG side-branch structures
Side-chains attach to the β-(1,4)-glucose backbone of xyloglucan and can vary dramatically in structure. Each side-chain structure is represented by a specific letter code following the nomenclature described by Fry *et al.* (1993). The most common side-branch structures are shown here but others do exist. For example, side-chains D and E are only found in *Equisetum* and various lycopodiophytes (Peña *et al.*, 2008).

Xyloglucan coats cellulose microfibrils and binds tightly through extensive hydrogen-bonding (McNeill *et al*., 1984; Hayashi *et al*., 1987). Extraction studies have shown xyloglucan molecules with an average length of around 200 nm, sufficient to span the 20-40 nm distance between cellulose microfibrils and act as a flexible tether between them (Pauly *et al*., 1999a). The rate and extent of cellular expansion is dictated by the dynamic interactions between cellulose and hemicellulosic polysaccharides, such as xyloglucan (Carpita & Gilbeaut, 1993). It has been observed that increased prevalence of cross-links between xyloglucan and cellulose correlates with a decrease in the strength and overall stiffness of the cell wall. The increased flexibility resulting from decreased cell wall rigidity allows greater extensibility and thus elongation in areas of turgor-driven growth, without compromising the cell's integrity by balancing strength and extensibility (Cosgrove, 1997; Whitney *et al.*, 1999). Xyloglucan has also been identified as the main seed-storage polysaccharide in a number of angiosperms (Kooiman, 1960; Wang *et al*., 1996).

1.1.2.2 Mixed-linkage glucan

Mixed-linkage glucan (MLG) is an unbranched homopolysaccharide of β-D-glucanopyranosyl residues. The backbone contains rigid regions of β-1,4-linked cellulose units, typically cellotriose (DP3) and cellotetraose (DP4), connected by a single β-1,3-bond which acts as a flexible linker thus giving the polysaccharide a kinked appearance (Peat *et al*., 1957; Burton & Fincher, 2009). MLG is naturally occurring in the Poales (Popper & Fry, 2004), Equisetales (Fry *et al*., 2008b; Sørenson *et al*., 2008; Xue & Fry, 2012) and some lichens (Scheller & Ulvskov, 2010). There is phylogenetic variation in the proportion of β-(1,3)- : β-(1,4)-linkages and proportion of cellotriose : cellotetraose units (Woodward & Fincher, 1983). Poalean MLG has a greater proportion of cellotriose units than cellotetraose (Meikle *et al*., 1994), the ratio of which varies between species. For example, the ratio of DP3:DP4 in *Zea mays* is 1.5 compared to 4.5 in *Triticum aestivum* (wheat flour) (Bucheridge *et al*., 1999; Li *et al*., 2006). Meanwhile, in *Equisetum* MLG, cellotetraose units are the main component with significantly

fewer cellotriose units. There is also an appreciable amount of cellobiose (DP2) units (Figure 3) (Fry *et al*., 2008b).

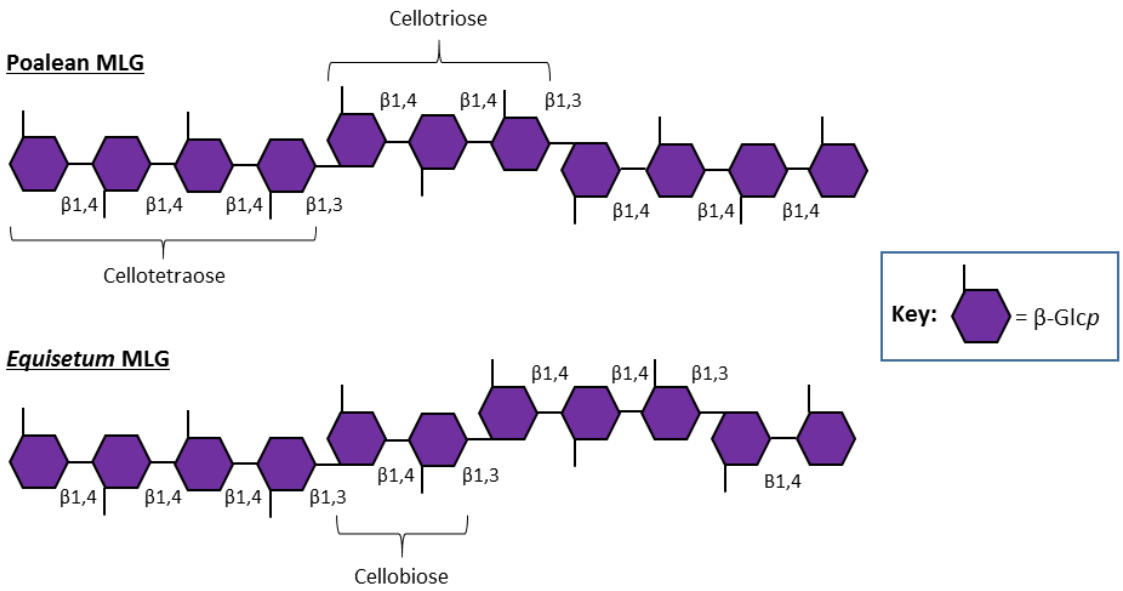


Figure 3: Poalean and Equisetum MLG

MLGs are of particular commercial interest due to their high concentrations in the PCW of economically relevant cereals such as rice, barley and wheat. Poalean MLG serves as a carbohydrate reserve which is hydrolysed to glucose following germination (Inouhe & Nevin, 1991). Meanwhile, *Equisetum* MLG is most prevalent in both young and senescing tissues with a proposed role in cell-expansion due to the presence of the cell-wall transglycanase, mixed-linkage glucan : xyloglucan *endo*transglucosylase (MXE, see 1.4.3) (Fry *et al*., 2008a; Sørenson *et al*., 2008).

A two-phase synthesis of MLG had been proposed where, initially, β-(1→4)-oligoglucosides were thought to be synthesised by cellulose synthase-like proteins (expressed by the *CslF* and *CslH* gene families) in the Golgi complex. These oligoglucosides were then thought to be transferred via exocytosis to the plasma membrane where modification of β-(1→4)-oligoglucosides in the apoplast by a callose synthase or XET would introduce β-(1→3)-linkages (Burton & Fincher, 2009; Doblin *et al*., 2009; Burton *et al*., 2010). However, the detection

of (1→3),(1→ 4)-β-D-glucans in the Golgi of developing maize coleoptiles by immunocytochemistry refutes this theory, instead proposing that MLG is synthesised within the Golgi like all other non-cellulosic cell-wall polysaccharides (Carpita & McCann, 2010).

1.1.2.3 Xylan

Despite xylan being the second most abundant hemicellulose in the PCW of dicots and non-graminaceous monocots, it is a quantitatively minor component compared to xyloglucan. By contrast, in the Poaceae, it is a major structural component of the PCW with glucuronoxylan constituting up to 40% of the dry weight (Darvill *et al*., 1980; Brummel & Schröder, 2009).

Xylans comprise a backbone of β-(1→4)-linked xylose residues which can be *O*-acetylated and/ or substituted at O-2 by α-Ara, α-GlcA and α-MeGlcA sidechains, giving rise to arabinoxylans, glucuronoxylans and glucuronoarabinoxylans (GAXs) (Ebringerová & Heinze, 2000; Johnston *et al*., 2013). Heteroxylans are structural components of the PCW that can act as cross-links between cellulose microfibrils by formation of hydrogen-bonds but these are weaker and slower developing than those observed with XyG or MLG (Carpita, 1983).

1.1.2.4 Mannan

Several different types of mannan have been classified in the PCW: pure mannans, galactomannans, glucomannans and galactoglucomannans (GGM). Pure mannans, which are composed of >95% mannose, and galactomannans, have a backbone of multiple β-(1→4)-linked mannose residues whilst the backbone of glucomannans and GGMs are interspersed with β-(1→4)-glucose residues (Wang *et al*., 2004; Scheller & Ulvskov 2010; del Carman Rodríguez-Gacio *et al*., 2012). With the exception of pure mannan, the mannan backbone can be interspersed with β-(1→4)-Glc residues and substituted with α-(1→6)-Gal sidechains which influence solubility, viscosity and interaction with other CW polysaccharides. An increase in glucose and/ or galactose residues in the mannan structure correlates

with an increase in solubility (Schröder *et al*., 2009; del Carman Rodríguez-Gacio *et al*., 2012).

Two main roles have been described for mannans: as structural components of the primary (and secondary) cell walls, and as storage polysaccharides in endosperm, vacuole and vegetative tissue cell walls (Matheson, 1990; Reid *et a*l., 2003; Handford *et al*, 2003; Schelle & Ulvskov, 2010).

Of particular interest to this study is the role of mannans, specifically glucomannans and GMMs, which are ubiquitous in the PCW of dicots and commelinoid monocots, as structural components of the PCW with their ability to form para-crystalline and cellulose tethering polymers, introducing flexibility into the cell wall in a manner reminiscent of XyG (Newman *et al*., 1994; Whitney *et al*., 1995, 1998). The formation of cellulose-glucomannan tethers in elongating maize coleoptiles (Carpita *et al*., 2001), and in differentiating tracheids during times of high water pressure (Hoosoo *et al*., 2002), indicate a potential role for mannans in wall remodelling during cell expansion.

1.1.3 Pectin

Pectin, the most structurally complex polysaccharide family, forms a matrix of anionic polysaccharides consisting of four main domains rich in α-D-GalA residues (up to ~70% of pectin): homogalacturonan, rhamnogalacturonan (RG) -I and -II, and xylogalacturonan (Mohnen, 2008; Popper & Fry, 2008). These domains interact via ionic bonds, borate ester cross-links and calcium bridges, allowing regulation of both the porosity of the PCW (Fleischer *et al*., 1999) and cell-cell adhesion (Jarvis *et al*., 2003). The vast array of structural epitopes within the pectin matrix impart unique functions upon it, implicating pectin in numerous roles including, but not limited to, plant development, growth and cell expansion, defense, morphogenesis, binding of ions and growth factors, cell-cell adhesion and cell signalling (Ridley *et al*., 2001; Mohnen, 2008). The wide range of potential roles also correlates with the extensive distribution of pectin in the plant. Pectin is abundant in a multitude of different areas including the middle

lamella, the walls surrounding growing and dividing cells, and also in the junctions between cells and secondary cell walls (Mohnen, 2008).

Pectin is synthesised in the Golgi, by approximately 67 different transferases, including glycosyl-, methyl- and acetyltransferases (Mohnen, 2008), before being transported to the cell wall in membrane vesicles (Nevenführ & Staehelin, 2001). It is thought that different pectic polysaccharides (i.e. RG-I, RG-II, etc) are covalently cross-linked, due to the harsh chemical or pectin-degrading enzyme treatments required to isolate them from one another. However, it is unknown how these polysaccharides are crosslinked. Originally it was proposed that homogalacturonan acted as the pectic backbone from which RG-I protruded as side chains. However, following further investigation the current consensus supports a structure of conjoined domains, linked end-to-end by glycosidic bonds between their backbone residues (Nakamura *et al*., 2002; Popper & Fry, 2008; Coenen *et al.*, 2007)

1.1.3.1 Homogalacturonan

Homogalacturonan is an unbranched chain of anionic α-GalA and uncharged MeGalA residues, which can be acetylated at O-2 or O-3, joined by α-(1→4) bonds (O'Neill *et al*., 1990) in stretches up up to 100 residues (Yapo *et al*., 2007) (Figure 4). Potential for other cross-linking esters of an unknown structure have also been proposed by MacKinnon *et al*. (2002). Homogalacturonan is the most abundant pectic polysaccharide, constituting ~65% of the total pectin (Mohnen, 2008).

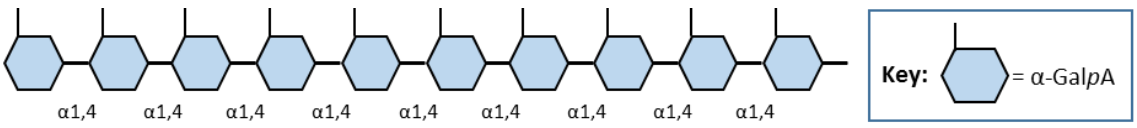


Figure 4: Homogalacturonan structure

Homogalacturonan has been implicated in the maintenance of correct cell adhesion *in vivo*. This is supported by observations with *quasimodo* mutants. *QUA1* encodes a putative glycosyltransferase and, following an insert mutation in the promoter of this gene, *Arabidopsis thaliana* presented a dwarf phenotype and a reduction in cell adhesion, resulting from a 25% decrease in homogalacturonan content (Bouton *et al*., 2002; Mouille *et al*., 2007). It has also been shown that an increase in flexibility results from a decrease in the homogalaturonan content of pectin (Ralet *et al*., 2008).

1.1.3.2 Xylogalacturonan

Xylogalacturon comprises a homogalacturonan backbone which is substituted at O-3 with a β-Xyl residue. This β-(1,3)-linked xylose side-branch is sometimes substituted at O-4 with another β-Xyl (Zandleven *et al*., 2006) (Figure 5). Xylogalacturonan is most prevalent in reproductive tissues but has also been detected in the stem and leaves of Arabidopsis (Zandleven *et al*., 2007). A proposed function for xylogalacturonan is for increasing the resistance of homogalacturonanan to degradation by endopolygalacturonase. Jensen *et al*. (2008) detected a biosynthetic gene that, following activation as a result of pathogenic attack and threat of subsequent endopolygalacturonase digestion, upregulated XG expression.

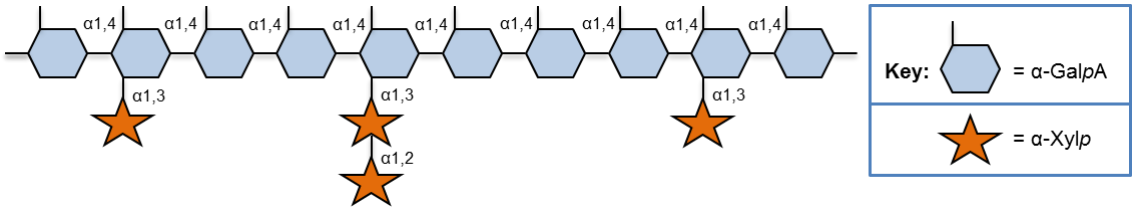


Figure 5: Xylogalacturonan structure

1.1.3.3 Rhamnogalacturonan-I

RG-I is the only pectin not built on a homogalacturonan backbone, instead having a backbone of alternating α-D-GalA and α-L-Rha residues (Figure 6). Approximately 20-80% (Mohnen, 2008) of α-L-Rha residues display branching of neutral polysaccharides – β-D-Gal and α-L-Ara (Lerouge *et al*., 1993; Fry *et al*., 2000) – and is hypothesised to covalently bind hemicelluloses thus integrating the main polysaccharide domains (Keegstra *et al.*, 1973; Fry, 1989; Ait Mohand & Farkaš, 2006). In fact, Thompson & Fry (2000) and Popper & Fry (2005) observed xyloglucan-pectin complexes accounted for 30-70% of the total xyloglucan in a range of angiosperm cell-suspension cultures.

RG-I has been implicated in a number of cell wall processes, including cell adhesion (Caffall & Mohnen, 2009), pathogen resistance (Wydra & Beri, 2007), pectin porosity and, as a result, control of the movement and activity of wall-metabolising enzymes (Carpita & Gibeaut, 1993), and water-retention (Naran *et al*., 2008). However, it has not been established how RG-I structure relates to function (Willats *et al*., 2001). It has been proposed that the size and shape of RG-I side chains may influence pectin properties. For example, changes in tissue firmness was observed in relation to degradation of RG-I arabinan and galactan side-chains (Brummel, 2006).

Antibody studies have detected arabinan and galactan epitopes in different regions of the cell wall, indicating a dynamic RG-I structure (Willats *et al*., 1998; Bush & McCann, 1999). Although arabinan and galactan RG-I side-chains can occupy the same cell, arabinan is most abundantly detected in meristematic cells whilst galactan is usually only detected in the cell wall after elongation (Yapo, 2011).

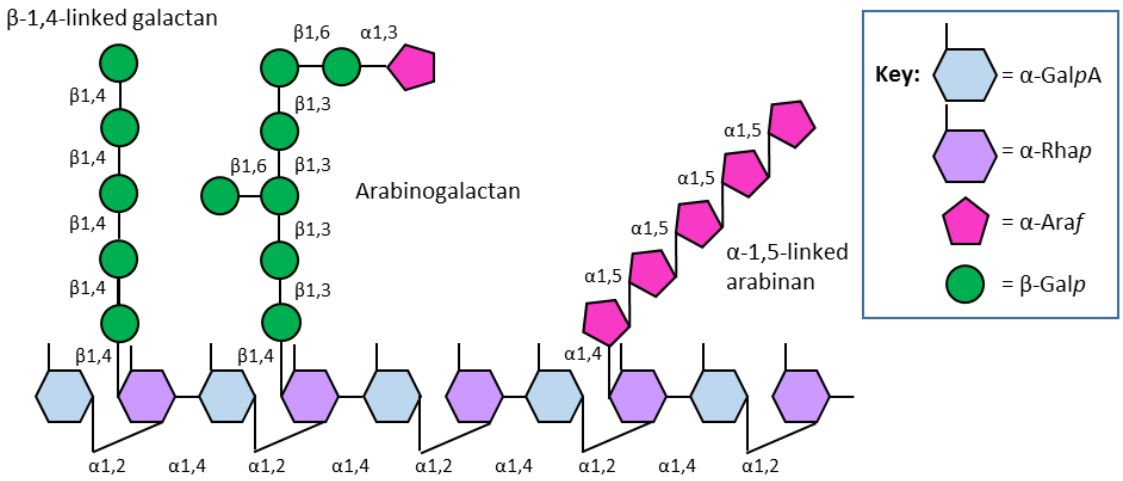


Figure 6: Rhamnogalacturonan-I structure

1.1.3.4 Rhamnogalacturonan-II

Although RG-II contains a homogalacturonan backbone, its extensive array of sidebranches consisting of 12 different monosaccharides and over 20 linkages (Mohnen, 2008) means that it is the most structurally complex pectin. RG-II constitutes ~10% of the total pectin (O'Neill *et al.*, 2004). Evolution of RG-II was associated with the adaption of land plants for upright growth and the development of a secondary cell wall (Matsunaga *et al.*, 2004). It has been proposed that RG-II plays an important structural role as it is widespread throughout the plant, resistant to degradation by all known pectin-degrading enzymes and is structurally conserved (Vidal *et al.*, 1999; Willets *et al.*, 2001).

1.2 Cell wall composition varies phylogenetically

PCW composition varies both chemically and physically from the phylogenetic to the cellular level allowing its adaptation to specific functions and precise regulation (Franková & Fry, 2011; Popper *et al.*, 2011). The cell wall of dicotyledonous and non-gramineous monocotyledonous plants are Type I while grass/ commelinids have Type II cell walls.

The major hemicellulose in Type I walls is XyG (Pauly *et al*., 1999a; O'Neill & York, 2003) which comprises around 20% of the dry weight of the PCW whilst pectin contributes approximately 30% (w/w) of the dry weight (Ridley *et al*., 2001), introducing strength and flexibility. By contrast, pectin content in Type II walls is less pronounced, whilst XyG content is often very low. For example, XyG content in barley PCW has been recorded as between 2-5% (w/w) of the total dry weight (Scheller & Ulvskov, 2010). In place of XyG, MLG (in the Poales and Equisetales (Fry *et al*., 2008b)), glucomannan and glucuronoarabinoxylan (GAX) fulfil similar functions as they are structurally analogous (Carpita & Gibeaut, 1993; Carpita, 1996). In Type II PCWs xylan is the main non-cellulosic polysaccharide (predominately arabinoxylan or GAX (Scheller & Ulvskov, 2010)). Differences in the fundamental structure of the PCW also suggest differences in specific enzymes, proteins and developmental signals between the two wall types.

Although generally applicable, this classification system is insufficient to describe primitive or evolutionarily isolated taxa which have cell-wall architectures that do not fit fully into either group. *Equisetum*, for example, is from an evolutionarily isolated genus and has a distinct cell wall composition. Whilst *Equisetum* contains high levels of MLG, a characterisitic Type II wall polysaccharide, it does not have sufficient levels of xylans and glucuronoxylans to be Type II. *Equisetum* cell walls also contain high levels of cellulose and pectin which is characteristic of Type I cells walls (Popper and Fry, 2004; Fry *et al*., 2007).

1.3 Cell wall enzymes

Currently known cell-wall enzymes – hydrolases, oxidoreductases and transferases – catalyse four classes of reactions: hydrolysis, transglycosylation, transacylation and redox reactions (Fry, 1995). Hydrolases predominately act on glycosidic bonds but have also been shown to act on esters (Tieman *et al*., 1992; Ferté *et al*., 1993) and peptide bonds (van der Wilden *et al*., 1983). Transglycanases, the class of transferases most pertinent to this study, are *endo*-acting enzymes that exhibit a high transglycanase : hydrolysis ratio even at low concentrations of acceptor substrate (Fry, 1995). Multiple enzyme classes have

been observed to participate in XyG metabolism including *endo*-1,4-β-D-glucanases, *exo*-glycosidases and XTHs (Fry, 2004).

1.4 Transglycanases and cellular expansion

Transglycanases catalyse the transfer of a non-terminal glycosyl group from a donor polysaccharide to an acceptor substrate (typically another polysaccharide molecule) and are thought to be involved in reversible loosening of the PCW, allowing cell-wall reconstruction and elongation (Fry *et al*., 1992; Darley *et al*., 2001). Expansion and reconstruction of the PCW are integral to many central processes of the plant including germination, fruit ripening, organ abscission, vascular differentiation and growth (Carpita and McCann, 2000). For example, in roots and leaves it creates a larger surface area for nutrient uptake whilst in stems it allows increased light intensity due to more advantageous positioning of leaves. It is highly regulated, initiated by a number of stimuli including stress, hormones (*e.g.* auxin) and developmental signals (Keegstra *et al*., 1973), and involves the reorientation of PCW components. It is thought that enzymatic cleavage of tethering molecules allows internal hydrostatic pressure to separate microfibrils thus expanding the cell. New microfibrils and associated polysaccharides are laid down on the innermost surface of the wall (Carpita & Gibeaut, 1993) so that, based on the multi-net growth hypothesis (Roelofsen & Houwink, 1953), the extended walls are comparable in strength and structure to the previously unextended wall.

1.4.1 Xyloglucan *endo*transglucosylase/ hydrolases

Xyloglucan *endo*transglucosylase/ hydrolases (XTHs) are a subfamily of the GH16 family of enzymes (see 1.6.1) (Cantarel *et al*., 2001) that are able to catalyse xyloglucan *endo*transglucosylase (XET) (EC 2.4.1.207) and xyloglucan *endo*-hydrolase (XEH) (EC 3.2.1.151) reactions (de Silva *et al*., 1993; Fanutti *et al*., 1993; Tabuchi *et al*., 2001; Rose *et al*., 2002). XTHs are apoplast-localised enzymes encoded by large multi-gene families. For example, *Arabidopsis thaliana* has 33 XTH coding genes allowing the synthesis of a diverse range of XTH

isozymes (Maris *et al*., 2011) which play intrinsic roles in processes integral to the viability of the PCW (Fry *et al*., 1992; Rose & Bennett, 1999) including cell wall morphogenesis (Cosgrove, 2005), storage polysaccharide mobilisation (de Silva *et al*., 1993) and loosening of the cell wall either reversibly (XET) or irreversibly (XEH) (Fry *et al*., 1992; Nishitani & Tominaga, 1992; Rose *et al*., 2002; Nishitani & Vissenburg, 2007).

Most XTHS for which full kinetic data are known are strict XETs which display little/ no detectable XEH activity (Fry *et al*., 1992; Nishitani & Tominaga, 1992). However, some XTHs are able to catalyse both XET and XEH reactions (Bourquin *et al*., 2002; Eklöf and Brumer, 2010). Structural analysis of XTHs has thus far been unable to elucidate what factor is the determinant for XET or XEH activity. A study by Baumann *et al*. (2007) indicated the presence of a unique extension of the loop connecting strands β8-β9 in predominant XEHs as a major (but not the sole) contributor to defining XEH over XET activity. This loop was found to lie adjacent to the active site in *Tm*-NXG1 – a GH16 *endo*-xyloglucanase with predominant XEH activity – and was capable of interacting with the substrate bound in the positive sub-sites of the binding cleft. Truncation of this loop resulted in diminished XEH activity and a significant increase in XET activity.

1.4.2 XET-active XTHs

XET activity is ubiquitous throughout land plants and was the first cell-wall acting transglycanase activity discovered in the PCW. XET catalyses the transfer of a xyloglucan glucosyl group, formed via the endolytic cleavage of the xyloglucan backbone, to the O-4 of the non-reducing terminal of a xyloglucan or a XGO and formation of a new β-(1,4)-glycosidic bond (Fry *et al*., 1992; Bourquin *et al*., 2002). XET activity, and potential novel transglycanase activities, are predominately studied *in vitro* using a donor polysaccharide and labelled oligosaccharide acceptor (Figure 7). XET activity and expression is detected at high levels in both growing tissues (Fry, 1992; Pritchard, 1993; Palmer & Davies 1996; Vissenburg *et al*., 2000, 2001) and in tissues where expansion has ceased (Arrowsmith & de Silva, 1995; Xu *et al*., 1995; Palmer & Davies, 1996).

Many roles have been proposed for XET *in vivo* including, but not limited to, restructuring of the PCW during secondary wall deposition (Bourquin *et al.*, 2002), cell-wall restructuring (Thompson & Fry, 2001), development of vascular tissues (Hernández-Nistal *et al.*, 2010) and cell wall assembly (Thompson *et al.*, 1997). XETs are encoded by large multi-gene families containing 20-60 genes (Elköf & Brumer, 2010) which likely reflects the functional, spatial and temporal differences observed between different isozymes, even within the same cell (Campbell & Braam, 1999a; Steele & Fry, 2000; Nishitani, 2005).

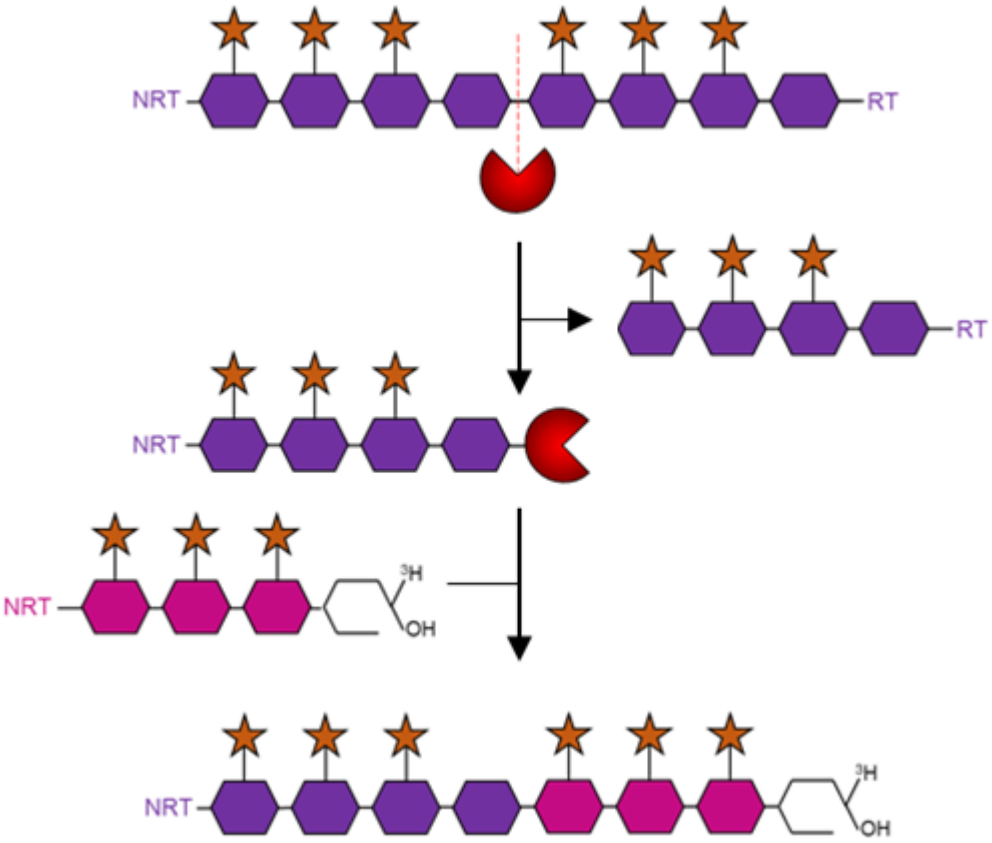


Figure 7: XET activity as a representative example of endotransglycosylation.
XTH (red) catalyses the cleavage of the backbone xyloglucan and subsequent grafting of a section of this xyloglucan donor (purple) to the non-reducing terminus (NRT) of a xyloglucan or XGO acceptor (pink). The reducing terminus (RT) of the acceptor polysaccharide can be tagged with a fluorescent or radioactive moiety, such as SR or ^{3}H (as shown) respectively, to allow identification of endotransglycosylation product.

Variation between XET isozymes is further exhibited by different substrate specificities. Some XETs are highly specific whilst others display a degree of promiscuity with respect to acceptor and donor substrates (Kosík *et al*., 2010; Maris *et al*., 2011). Maris *et al*. (2011) observed that for some *At*XTHs, although xyloglucan is the preferred donor substrate, some transglycanase activity can occur with other donor substrates such as hydroxyethylcellulose (HEC) and water-soluble cellulose acetate (WSCA) whilst a barley XET isozyme displayed very low hetero-transglyanase activity between a mixed-linkage glucan donor and xyloglucan oligosaccharide acceptor (Hrmová *et al.*, 2007). Substrate specificity can also be dependent on specific branching patterns or the length of the donor/ acceptor substrate. For example, *Va*XTH1 from adzuki beans (*Vigna angularis*) only acts efficiently as a transglycanase using donors > 10 kDa in length (Nishitani & Tominaga, 1992). Other studies (Fanutti *et al*., 1993, 1996; Schröder *et al*., 1998; Saura-Valls *et al*., 2008) have shown that with other isozymes, oligosaccharides are sufficient to function as donors.

The most important function of XET is its probable role in expansion and elongation of the cell wall via controlled PCW-loosening, proposed by Fry *et al.* (1992) following the observation that there was a negative correlation between XET activity and cell age; XET activity is highest in growing stems (Fry, 1995). This was supported by observations of a positive correlation between XET activity and cell-wall extensibility (Pritchard *et al.*, 1993). XET catalyses the endolytic cleavage of XyG tethers between cellulose microfibrils allowing them to separate and for the cell wall to undergo turgor-driven expansion (Johansson *et al*., 2004) before rapid reinforcement of the PCW via grafting of the new donor polysaccharide reducing terminal (RT) to the NRT of an acceptor polysaccharide with no net loss of cell wall strength (Smith and Fry, 1991; Xu *et al*, 1995; Purugganan *et al*., 1997; Campbell & Braam, 1999a). The 3D-structure and catalytic mechanism of XETs are discussed in detail in sections 1.6.1.1 and 1.6.1.2 respectively.

1.4.3 Mixed-linkage glucan : xyloglucan *endo*transglucosylase

For a long time it was thought that only homo-transglycanases were active in the PCW of land plants. However, the subsequent discovery of the hetero-transglycanase mixed-linkage (1→3, 1→4)-β-D-glucan (MLG) : xyloglucan *endo*transglucosylase (MXE) in *Equisetum* highlighted the potential for a range of novel homo- or hetero-transglycanases. MXE, although capable of homo-transglucosylation with a xyloglucan donor and xyloglucan-based acceptor (KE Mohler & TJ Simmons, unpublished data), preferentially catalyses the reaction between an MLG donor and a xyloglucan (or xyloglucan oligosaccharide) acceptor (Fry *et al.*, 2008a) and is currently the only known predominant hetero-transglycanase in the PCW.

Significant MXE activity observed within land plants is unique to *Equisetum*, although an MXE-like activity has been reported as a side-reaction of some XTHs. Hrmová *et al*. (2007) observed a barley XTH with MXE activity of ~0.2% (of the XET activity) in the presence of a MLG donor and SR-tagged XGO acceptor. A similar level of MXE-like activity was observed in a crude *Holcus lanatus* extract (0.17% of XET activity) (Fry *et al*., 2008a). By contrast, MXE activity in *Equisetum* was significantly higher, producing activity correlating to 65% or more of the XET activity (Fry *et al*., 2008a).

Various characteristics of the MXE activity observed in *Equisetum* indicate that, rather than being a side-reaction of an XTH, it is due to the presence of a different protein (Fry *et al*., 2008a). For example, in *Equisetum* the MXE : XET ratio varies both between species and seasonally within a species. MXE activity peaks in old *Equisetum* stems whilst XET is higher in young stems (Fry *et al*., 2008a) suggesting a potential role for MXE in strengthening and repair of mature cell walls rather than in cell elongation and growth like XET.

Further evidence supporting the hypothesis of seperate MXE and XET enzymes in *Equisetum* comes from analysis of their oligosaccharide acceptor-substrate preferences and enzyme kinetics. The acceptor substrate preference of *Equisetum* XET is similar to that recorded for dicots (Rose *et al*., 2002; Steele &

Fry, 2000), where XLLGol>XXXGol>XXGol. However, MXE has a significantly higher specificity for XXXGol over both XLLGol and XXGol. Estimated values of K_M for XXXGol for MXE and XET of 3.7± 1.5 μM and 80 μM respectively (Fry *et al*., 2008a), where the lower K_M of MXE for XXXGol indicates it has a higher affinity for this acceptor than XET. The V_{max}, which is the maximal rate of reaction an enzyme can achieve, of XET was 2 pmol h^{-1} with 1.4 μM XXXGol and rose to 26 pmol h^{-1} at 200 μM XXXGol. The V_{max} for MXE was consistently ~7 pmol h^{-1} at both concentrations of XXXGol which implies an extremely high affinity of MXE for XXXGol (Fry *et al*., 2008a). Donor substrate specificity also varied between MXE and XET in *Equisetum* with a K_M of 0.35 mg ml^{-1} for XyG and 4 mg ml^{-1} for barley MLG (Fry *et al*., 2008a). The donor K_M reported for *Equisetum* MXE is significantly lower than for any published XTH which has a range of K_M = 33-320 μM (Fry *et al*., 2002; Schröder *et al*., 1998; Takeda *et al*., 1996). Interestingly, low but significant levels of homo-transglycanase activity have been detected with an MLG-heptasaccharide acceptor and MLG donor polysaccharide (Fry *et al*., 2008a).

A potential commercial use of MXE would be in the genetic engineering of cereals, which possess MLG but lack MXE, to allow expression of *Equisetum* MXE. The consequence of MXE expression on the mechanical properties of the crop is unknown but it could potentially have a role similar to that of MXE in Equistetum where it is proposed to be involved in the strengthening and repair of mature cell walls. However, due to the differing MLG structures between the Poales and *Equisetum* (see 1.1.2.2) activity may differ between species. Increased MXE activity has been shown to correlate positively with increased cellotetraose content and is inactive with lichenan; a MLG composed almost entirely of cellotriose units (Fry *et al.*, 2008a).

1.4.3.1 Cellulose : xyloglucan endotransglucosylase

A potential novel transglucanase activity, cellulose : xyloglucan endotransglucosylase (CXE), has been detected in crude *Equisetum* enzyme extracts using a cellulose donor and XGO acceptor (KE Mohler & TJ Simmons, unpublished data). This is perhaps unsurprising giving the structural similarities

between cellulose, XyG and MLG. However, CXE activity has not been detected at significant levels in species other than *Equisetum*, thus coinciding with MXE. It is hypothesised that CXE activity is a side reaction of MXE activity.

1.4.4 Mannan *endo*transglycosylase

A mannan *endo*transglycosylase (MET) that exhibits trans-β-mannanase activity was identified by Schröder *et al.* (2004) *in vitro* under physiological conditions. High MET activity has been observed in tomato, limited to the skin and outer pericarp, with activity increasing from the green to ripe red tomato fruit stages before decreasing during over-ripening. A similar pattern of expression was observed in *Actinidia* flower buds with high MET expression during ovule formation and flower development before decreasing to undetectable levels when flower senescence began (Schröder *et al.*, 2004). High MET activity correlates with times of cellular expansion, in a manner similar to XET, thus suggesting MET may have a role in the structural reorganisation of the cell wall during growth and ripening.

MET has specificity for mannan-based polysaccharides containing a β-(1→4)-linked backbone – pure mannan, GMM, galactomannan and glucomannan – as a donor substrate but displays no preference between them (Schröder *et al.*, 2004). It is possible, due to the lack of preference for a specific mannan-based donor polysaccharide, that two different mannans could participate in a transglycanase reaction. Acceptor requirements, although not fully understood, are less flexible with a minimal requirement for a substituted/unsubstituted mannose residue (rather than glucose) at position 2 and an unsubstituted mannose residue at position 4 (counting from the NRT) (Schröder *et al.*, 2004). In addition, acceptors with longer backbones are prefered.

Interestingly, in tomato, MET activity, localisation and expression co-ordinated with that of *endo*-β-mannanase (Bewley *et al.*, 2000; Bourgault *et al.*, 2001). Expression of LeMAN4a, a retaining *endo*-β-mannanase, showed this enzyme to have the propensity to catalyse both hydrolysis and transglycosylation of PCW mannans (Schröder *et al.*, 2004, 2006). It has been speculated that MET and *endo*-

β-mannanase may result from different isoforms of the same protein exhibiting different catalytic processes as the result of varying post-translational modifications (Schröder *et al.*, 2004, 2006, 2009).

1.4.5 Trans β-xylanase

The most recently discovered transglycanase was described by Franková & Fry (2011) who observed trans-β-xylanase activity which catalysed the transglycanase reaction $2Xyl_6 \rightarrow Xyl_3 + Xyl_9$ in a variety of genera including *Marchantia*, *Selaginella* and *Equisetum*. Johnston *et al*. (2013) have since detected high levels of trans-β-xylanase in numerous fruits, imbibed seeds and in the PCW of cereal seedlings undergoing rapid growth. The highest activity observed was activity in papaya (*Carica papaya*). Trans-β-xylanase activity is reported to peak during times of rapid growth and fruit ripening (Johnston *et al*., 2013), suggesting a role in the rearrangement or remodelling of cellulose-associated heteroxylans in the PCW.

A range of heteroxylans, including arabinoxylan (wheat), glucuronoxylan (birchwood) and heteroxylans purified from the PCW of tomato and papaya were tested for their potential to act as suitable donor substrates for trans-β-xylanase. Although activity was observed with all donor heteroxylans tested, trans-β-xylanase preferentially catalysed reactions with donor substrates containing highly-substituted xylan backbones (Johnston *et al*., 2013; Ratke, 2014).

Like XET and MXE, trans-β-xylanase is a retaining enzyme that shows significant differences in expression patterns, both spatial and temporal, from its hydrolase counterpart, *endo*xylanase (Johnston *et al*., 2013; Ratke, 2014). Further research is required to determine whether trans-β-xylanase activity is due to the presence of a distinct enzyme, a different isoform of an *endo*xylanase or is the result of a side reaction of an alternative enzyme.

1.4.6 Novel transglycanases

Cell wall remodelling enzymes have been shown to have extensive variability in phylogenetic presence, substrate specificity and preference for homo- or hetero-transglycosylation suggesting the potential for a wealth of additional enzymes displaying transglycanase activity which remain, as yet, undiscovered.

Although, at present, transglycanases have been detected *in vitro* acting on all major PCW hemicelluloses, the possibility of other transglycanases remains high. Observations of inter-polymeric links in the PCW, such as that of "pectic-xylan–xyloglucan" complexes in cauliflower (Femenia *et al.*, 1999), covalent bonding between xyloglucan and acidic polysaccharides in angiosperms (Popper and Fry, 2005) and (1→4)-β-D-glucan linkages to pectin–xyloglucan complexes (Abdel-Massih *et al.*, 2003) indicate potential hetero-transglycanase activity; an activity currently only observed with MXE.

Of particular commercial interest is the potential for new transglycanase activities in the PCW of poacean crops. There is an incongruity between their low XyG content (Scheller & Ulvskov, 2010) and large number of putative XTH genes encoded within poacean genomes (Elköf & Brumer, 2010). Maintenance of these genes seems nonsensical unless the encoded putative XTHs are capable of catalysing significant levels of transglycosylation with non-XyG substrates.

1.5 *Pichia pastoris* expression systems

With the progression of plant science into the 'post-genomics' era, a requirement for heterologous expression systems allowing synthesis of functional proteins from specific encoding DNA has arisen (Hrmová & Fincher, 2009). Unfortunately, progression in this research has been hindered by the lack of convenient and consistently effective expression systems for the synthesis of active plant enzymes. Common problems include expression of an inactive protein (if the protein is expressed at all), production of a protein that lacks appropriate post-translational modifications and protein synthesis at an insufficiently high concentration to allow further biochemical and/ or structural analysis.

Of the expression systems currently available, the eukaryote *Pichia pastoris* has proven among the most effective in the production of plant XTHs with successful expression of genes from cauliflower (Henriksson *et al.*, 2003), tomato (Catala *et al*., 2001; Chanliaud *et al*., 2004) and nasturtium (Baumann *et al.*, 2007; Chanliaud *et al*., 2004) to name a few. *P. pastoris* is a methylotrophic yeast which is easy to genetically manipulate, can be grown to high cell densities and can perform post-translational modifications considered to be central to XTH function, such as *N*-glycosylation (Daly & Hearn, 2005; Johansson *et al*., 2004). In addition, the levels of endogenous proteins secreted from *P. pastoris* are relatively low (Daly & Hearn, 2005).

Although XTH genes have previously been expressed in heterologous *Escherichia coli* expression systems, this is not suitable for the production of functional proteins if they require post-translational modifications, such as *N*-glycosylation, phosphorylation, disulphide isomerisation, lipidation or sulphation, as further protein solubilisation and refolding procedures would be required. Extensive post-expression processing is slow, convoluted and results in a low yield of active enzyme.

1.5.1 Advantages of *Pichia pastoris* strain SMD1168H

The heterologous expression system in this project will be established via transformation of a pPICZαA : gene insert plasmid into *P. pastoris* strain SMD1168H. The pPICZαA plasmid (Invitrogen by Life Technologies, CA, USA) contains a functional alcohol oxidase promoter (5'*AOX1*) which is responsible for expression of the recombinant proteins when activated by the presence of methanol (MeOH) (Daly & Hearn, 2005). The presence of an AOX1 promoter allows greater control of this system. Both proliferation of *Pichia* to a sufficiently high density and expression of the recombinant protein can be controlled through use of media including glycerol and methanol respectively.

The α-factor secretion signal allows secretion of recombinant proteins from *Pichia*, making collection of the desired protein easier (Invitrogen, 2010). The α-factor is subsequently cleaved following successful secretion. Recombinant

proteins contain both a *c-myc* epitope and His-tag (6x) which allows easy detection via antibodies and purification. The pPICZαA plasmid also contains a *Sh ble* gene which produces a protein that confers resistance to zeocin, allowing selection of colonies containing the pPICZαA insert (Invitrogen, 2010).

1.6 Glycoside hydrolases

Glycoside hydrolases (GHs) are a group of 'Carbohydrate-Active Enzymes' (CAZy) that hydrolyse glycosidic linkages in both polysaccharides and *O*-, *N*- and *S*-linked glycosides. Currently, more than 100 GH families have been described and can be *endo*- or *exo*-acting. *Endo*-acting GHs cleave a glycosidic bond within the main chain of the polysaccharide whilst *exo*-acting GHs cleave a terminal glycosyl residue, usually from the NRT (Figure 8).

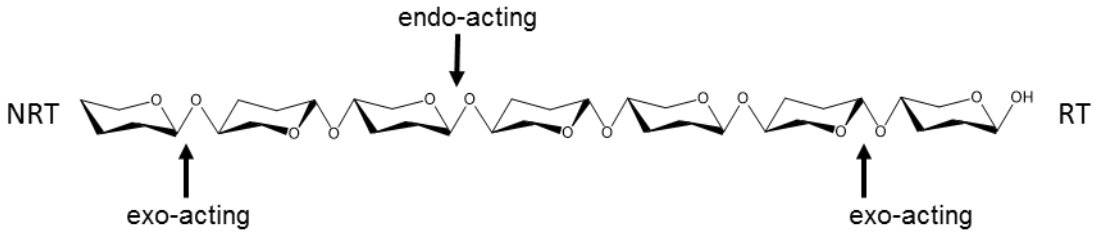


Figure 8: Sites of cleavage for *endo*- and *exo*-acting enzymes

Enzymes are grouped into families according to conservation of a common active site topology, substrate specificity and mode of catalysis. Grouping is independent of their phylogenetic distribution over eubacteria and eukaryotes (Barbeyron *et al*., 1998). It has been shown, via high resolution structural data, that proteins within the same GH group have a conserved core including active site residues and major element of secondary and tertiary structure even when primary structure similarity is low (Gebler *et al*., 1992). In addition, the molecular mechanism within most GH families is conserved with the group adopting either an inverting or retaining mechanism (see 1.6.1.2). A notable exception of this is the GH97 family which can adopt both mechanisms; the inverting mechanism is conducted through an active site glutamate residue acting as a general base whilst the retaining mechanism uses an aspartate residue that acts as a catalytic

nucleophile (Gloster *et al*., 2008). GH families are often grouped into one of fourteen clans – from GH-A to GH-N – based on the conservation of a common 3D-structure and catalytic machinery regardless of low primary sequence identity or differences in substrate specificity.

In addition to GHs, CAZy also comprises over 90 families of glycosyltransferases (GTs). GTs have been implicated in polysaccharide synthesis as they catalyse the formation of a glycosidic bond between an active donor (i.e. a saccharide donor containing a nucleoside or lipd phosphate) and a specific acceptor to form a glycoside (Lairson *et al*., 2008). XET was originally classified as an *endo*-GT (Nishitani & Tominaga, 1992) but has been reclassified as a GH due to structural analysis and the enzyme's dual-functionality.

1.6.1 GH16 family

Most pertinent to this study is the GH16 family of enzymes, a group with an extensive variety of substrate specificities leading to cleavage of β-1,3- or β-1,4-glycosidic bonds in various glucans and galactans. Substrate specificity of GH16s is amongst the most varied of any GH group with enzyme classes including xyloglucan : xyloglucosyl transferases (XET, EC 2.4.1.207), keratin-sulphate *endo*-1,4-β-galactosidases (EC 3.2.1.103), xyloglucan-specific *endo*-β-1,4-glucanases (i.e. XEG, EC 3.2.1.151), *endo*-1,3-β-galactanases (EC3.2.1.-), *endo*-1,3-β-glucanases (laminarinase, EC 3.2.1.39), *endo*-1,3(4)-β-glucanases (EC 3.2.1.6), lichenases (EC 3.2.1.73), β-agarases (EC 3.2.1.81), β-porphyranases (EC 3.2.1.178) and κ-carageenanases (EC 3.2.1.83) (Carbohydrate-Active Enzyme (CAZY) server – www.cazy.org). Despite the wide differences in substrate specificities between GH16 members, phylogenetic evidence indicates that all GH16 enzyme classes evolved from a common ancestor which had laminarinase activity (Michel *et al*., 2001). GH16 is a member of the GH clan-B along with the closely related GH7s which include cellobiohydrolases (EC 3.2.1.176), chitosanases (EC 3.2.1.132) and endoglucanases (EC 3.2.1.4 and EC 3.2.1.73). All known XTH and XET-like enzymes are members of the GH16 family although

transglycanase activities have been observed in other GH families including, but not limited to, GH5 (trans-β-mannanase), GH12 and GH17.

GH16 enzymes are separated into two subgroups – GH16a and GH16b – dependent on their active site motif. While GH16a enzymes contain the ancestral β-bulge (consistent with the GH7 β-(1→4)-D-glucanases), GH16b enzymes contain a regular β-strand active site (for further information see 1.6.1.1). Subgroup GH16b contains XTHs, lichenases (mixed-linkage (1→3),(1→4)-β-D-endogluanases) and Crh enzymes (which catalyse fungal chitin : laminarin endotransglycosylation (Cabib *et al.,* 2008)).

Originally, the molecular phylogeny of XTHs and XTH gene products were divided into three clades: I, II and III (Campbell & Braam, 1999b). However, these clades have since been revised due to an ever-increasing wealth of biochemical and genetic information (Rose *et al*., 2002). Although originally distinct, when Yokoyama *et al.* (2004) compared the genomes of rice (*Oryza sativa*) and Arabidopsis (*Arabidopsis thaliana*) they found that clades I and II had become indistinguishable. Clade I/II contains the majority of XTHs and it has been suggested by Miedes *et al*. (2011) that XTHs from this group are involved in restructuring during cell wall growth and development. Clade III has been separated into 2 groups based on catalytic measurements and sequence analysis. This distinction is important as, while clade III-A is thought to be the only XTH clade to contain predominately hydrolytic members (Eklöf & Brumer, 2010), heterologously expressed proteins from clade III-B show predominately or exclusively XET activity (Campbell & Braam, 1999a; Saladie *et al*., 2006; Kaewthai *et al.*, 2010).

1.6.1.1 GH16 structure and that of XTHs

Structural analysis of GH16 proteins have shown them to have a β-jelly-roll fold structure composed of two anti-parallel β-sheets which stack to form a β-sandwich consisting of one convex and one concave face (Figure 10) (Johansson *et al.*, 2004). Crystallographic studies have revealed an identical folding topology in the catalytic binding domains of both GH16 (Keital *et al.*, 1993; Michel *et al.*,

2001) and the closely related GH7 enzymes (Divne *et al*., 1994). Although variations in the primary structure of XETs does not seem to significantly alter their conserved secondary structures, even small difference in primary structure can significantly alter their biochemical properties (e.g. optimum temperature, K_M, etc.). Campbell & Braam (1999a) studied four *Arabidopsis* XTHs with different amino acid sequences and concluded that each XTH differed in XET activity dependent on pH, temperature and substrate.

Observations on the X-ray structure of *Ptt*XET16-34 suggest that the conserved active site of XETs have four –ve ('donor-binding') and three +ve subsites ('acceptor-binding') (based on the notation by Davies *et al*., 1997) (Saura-Valls *et al*., 2008) and it is the loops that connect the β-strands within the core of the enzyme that govern the shape and characteristics of the donor and acceptor substrate binding sites. When the donor polysaccharide enters the binding cleft a conformational change occurs resulting in a shift of approximately 1 Å in the loop connecting strands β13 and β14 (Johansson *et al*., 2004). This shift results in constriction of the binding site and stacking of donor polysaccharide glycosyl rings.

The substrate specificity of the +ve and -ve sub-sites within the binding cleft differs significantly. The requirements for the –ve sub-sites are more relaxed than those of the +ve sub-sites (Fry *et al*., 1992; Fanutti *et al*., 1993, 1996; Saura-Valls *et al*., 2008). The minimal requirement of the –ve sub-sites is a cellobiosyl (GG) moiety in -1 and -2 but there is an absolute requirement that the glycosyl-ring in sub-site -1 is unbranched. This is important as the 6-OH of the glycosyl-ring in sub-site -1 hydrogen-bonds with the general acid/ base residue of the active site whilst incorporation of a larger moiety is impossible due to close protein contacts (Fanutti *et al*., 1993, 1996; Saura-Valls *et al.*, 2008). As a result of the more relaxed substrate specificity for the donor polysaccharide, any glucosidic bond following an unbranched glucosyl unit within the main chain can occupy sub-site -1 thus allowing XET to cleave the donor polysaccharide to produce various XGOs (Saura-Valls *et al*., 2008). Sub-sites -2 and -3 are involved in transition state stabilisation but, contrary to research by Fanutti *et al.* (1993, 1996), it has been found that Xyl

substitution of the glycosyl-ring at position -3 is not essential (Saura-Valls *et al*., 2008). By contrast, the +ve sub-sites are more specific with a minimal structural requirement for activity of XXG, of which the X at sub-site +1 is absolute requirement. An X at subsite +2 is not essential but is preferential as it contributes to transition state stability (Fry *et al*., 1992; Fanutti *et al*., 1993, 1996; Saura-Valls *et al*., 2008).

The active site residues of GH16s are contained within a single β-strand – β7 in XETs – on the concave face of the β-sandwich at the centre of the binding cleft (Ståhlberg *et al*., 1996). Two signature active-site motifs are found within clan-B GHs, the ancestral β-bulge (EXDXXE) and the regular β-strand (EXDXE), the latter is found in XETs (Michel *et al*., 2001). The catalytic components of the active site motif are the first and last glutamic acid (E) residues which confer the role of catalytic nucleophile and general acid/ base respectively (Keitel *et al*., 1993; Hahn *et al*., 1995; Ståhlberg *et al*., 1996). The central aspartic acid (D) residue of the motif allows formation of hydrogen-bonds with the catalytic nucleophile following insertion of the donor polysaccharide in the binding cleft (Johansson *et al.*, 2004). Discovery of the catalytic nucleophile resulted from non-specific epoxyalkyl β-glycoside inhibitor assays on an *endo*-1,3-1,4-β-D-glucan 4-glucanohydrolase from *Bacillus amyloliquefaciens* (Høj *et al*., 1992; Viladot *et al*., 1998) whilst the general acid/ base residue was identified in 1,3-1,4-β-D-glucan 4-glucanohydrolase from *Bacillus lichenformis* by azide rescue of an inactivated E138A mutant (Viladot *et al*., 1998).

XTHs are unique from most other known GH16s, which have their C-terminal located at the end of β14, due to the presence of a C-terminal extension which forms the only α-helix and an additional β-strand (β15). The biological significance of this extension has not been elucidated but it has a proposed role in the functioning of the enzyme due to conserved disulphide bonds in XETs between it and strands β14 and β15. The extension is also thought to be involved in extending the length of the binding cleft (Johannson *et al.*, 2004).

Encapsulating the binding cleft of GH16 enzymes is an array of aromatic residues that mediate formation of van der Waals interactions between the enzyme and docking substrates. Many of these aromatic residues are conserved within clan-B (Michel *et al*., 2001), such as Y75, which was identified in *Ptt*XET16A and is integral in the co-ordination of the glucose residue in sub-site -1 during attack by the catalytic nucleophile (Johansson *et al.*, 2004). However, it is the aromatic residues which are unique to each enzyme class that give insight into their differing substrate specificities. For example, in *Ptt*XET16A the aromatic residues Y170 and Y250 are thought to be unique to XETs with a proposed role in the specific recognition of XyG (Johansson *et al*., 2004).

No counterpart has been found in XETs of the common GH16 Ca^{2+} binding site located on the convex face of the β-sandwich of other GH16 proteins (Keitel *et al*., 1994; Michel *et al*., 2001; Johansson *et al*., 2004). However, unlike other GH16s, most XETs studied have a conserved glycosylation site 5-15 residues from the active site towards the RT (Johannson *et al*., 2004) which has been proven to be vital for XET function. Multiple studies have reported that deglycosylation of this residue results in the loss of XET activity (Campbell & Braam, 1999a; Henriksson *et al*., 2003). However, deglycosylation of this residue in *Ptt*XET16A resulted in retention of significant XET activity (Johansson *et al*., 2004) suggesting *N*-glycosylation is not an absolute requirement for XET activity.

1.6.1.2 Mechanism of GH16 activity in the context of XET

GH enzymes can be either inverting or retaining. Inverting enzymes hydrolyse polysaccharides via a one-step, single-displacement mechanism, resulting in the net inversion of the anomeric carbon. Meanwhile, retaining enzymes act through a two-step, double displacement mechanism which leads to the net retention of the anomeric carbon configuration via the formation of a glycosyl-enzyme intermediate (Koshland, 1953). NMR analysis by Malet *et al*. (1993) of a *Bacilus lichenformis* GH16 *endo*-1,3-1,4-β-D-glucan 4-glucanohydrolase indicated the use of a retaining mechanism and this has been shown to be consistent though the

family. All known XETs, transglycanases and other GH16 proteins work through a retaining mechanism.

XET is thought to act via a Ping-Pong Bi Bi mechanism; a bi-substrate system resulting in the formation of two reaction products (Figure 9). Transglycanase activity is mediated by carboxylic amino acids located approximately 5.5 Å apart in the active site of the enzyme that act as a catalytic nucleophile and a general acid/base (Saura-Valls *et al.*, 2008).

Initially glycosylation occurs via attack on the anomeric carbon by the catalytic nucleophile and subsequent protonation of the glycosidic oxygen as the bond is cleaved by the general acid residue. A stable glycosyl-enzyme intermediate is formed. Next, deglycosylation occurs as the glycosyl-enzyme intermediate undergoes attack by a molecule of XyG (or XGO) which has been deprotonated by the general base residue thus forming a new transglycanase product which is released from the enzyme binding cleft (Saura-Valls *et al.*, 2008).

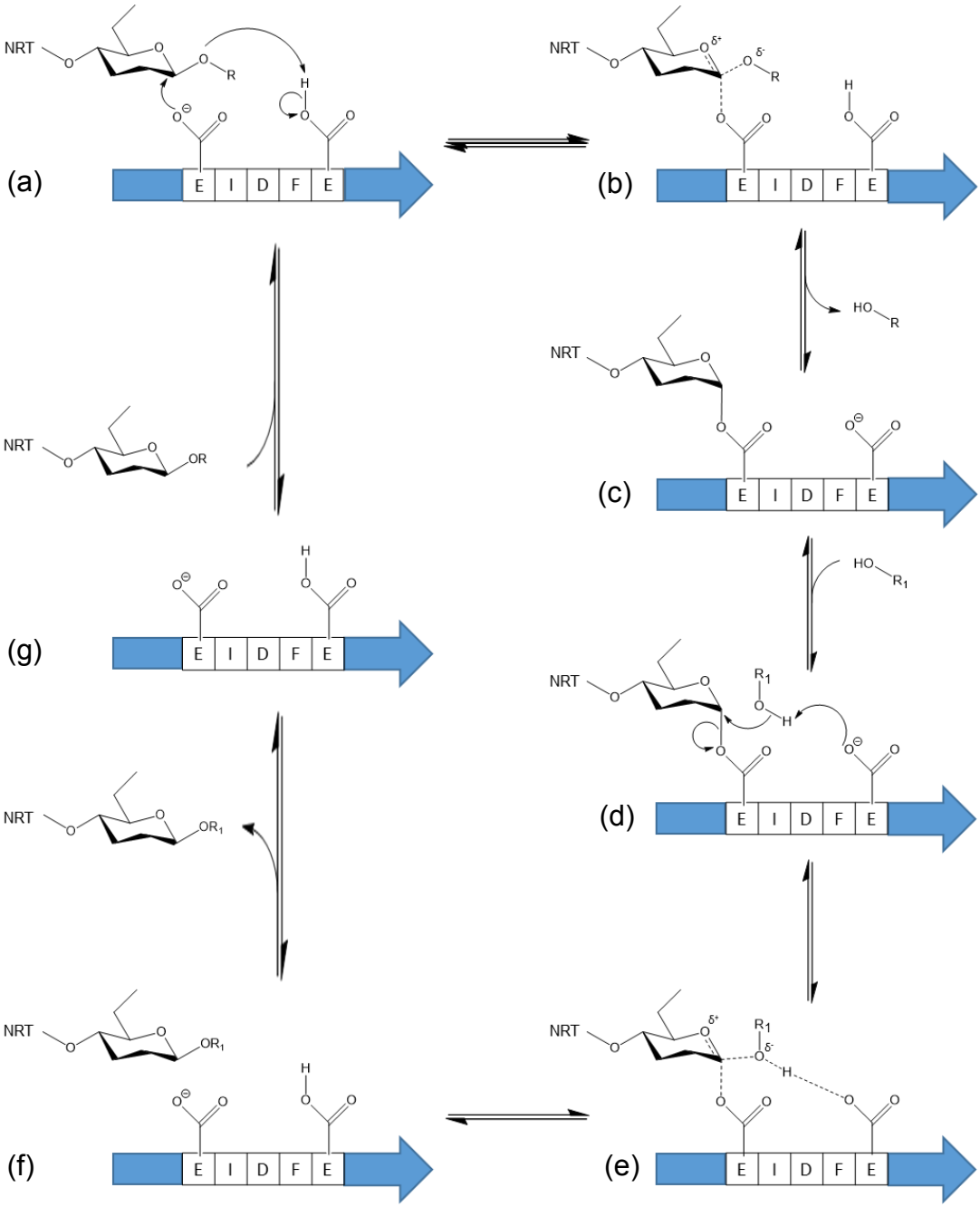


Figure 9: Ping-Pong Bi Bi mechanism of XET activity
Once a donor polysaccharide enters the XET binding cleft, (a) the catalytic nucleophile attacks the anomeric carbon of an unsubstituted glucose residue and leads to (b) protonation of the glycosidic oxygen and cleavage of the glycosidic bond by the general acid residue. This results in (c) the formation of a stable glycosyl-enzyme intermediate. Occupation of the positive sub-sites (d) with an acceptor substrate that attacks the glycosyl-enzyme intermediate leads to (e) deprotonation of the general base residue. This results in the formation of (f) a novel transglucanase product which exits the binding cleft allowing the (g) unoccupied active site to catalyse further transglucanase reactions. R = donor polysaccharide reducing terminal, R_1 = acceptor oligo/ polysaccharide reducing terminal.

1.6.2 GH17

The GH17 family, a member of GH clan-A, contains laminarinases (EC 3.2.1.39), 1,3;1,4-β-D-glucan endohydrolases (EC 3.1.2.73) and a 1,3-β-D glucan *exo*hydrolase (lichenases, EC 3.1.2.58). Despite enzyme class similarities between GH16 and GH17, with regard to a common ancestor with laminarinase activity, the presence of laminarinases, and catalysis in both families occurring via the Koshland retaining mechanism (Koshland, 1953; Chen *et al.*, 1995), the enzymes from each family are distinct in amino acid sequence and secondary structure.

GH17 enzymes have an α/β-barrel structure (Figure 10) containing an extensive open binding cleft capable of accommodating 6-8 glycosyl residues (Varghese *et al.*, 1994). In accordance with other clan-A GHs, the catalytic nucleophile and general acid/ base have been observed on strands β7 and β4 respectively (Varghese *et al*., 1994; Henrissat *et al*., 1995; Pickersgill *et al*., 1998). The catalytic nucleophile was identified as Glu232 in barley 1,3;1,4-β-D-glucan *endo*-hydrolases and Glu231 in 1,3-β-glucanases via epoxyalkyl β-oligosaccharide labelling, indicating conservation of this residue throughout the family (Chen *et al*., 1993). The general acid/base residue was proposed to be Glu93 (Pickersgill *et al*., 1998), which resides approximately 5.5 Å from the catalytic nucleophile, correlating with the distance between active residues that is characteristic for retaining enzymes.

1.6.3 GH64

Although little research has focussed on the GH64 family, it is known to contain laminarinases (EC 3.2.1.39) which are significantly different from those found in families GH16 and GH17. Known GH64 laminarinases have been described as inverting enzymes and it remains unclear as to whether they are *endo*- or *exo*-acting (Davies & Henrissat, 1995; Nishimura *et al*., 2001). Possibly the best characterised GH64 is laminaripentaose-producing β-1,3-glucanase (LPHase) from *Streptomyces matensis* DIC-108. This protein consists of a β-barrel domain (β-strands 1-10) and contains a mixed (α/β) domain. Each exterior face of the α/β is covered by a β-sheet to form a novel crescent-like fold (Figure 10) (Wu *et*

al., 2009). It is proposed that two carboxylates - Glu154 and Asp170 – act as proton donor and general base respectively during hydrolysis (Wu *et al*., 2009; Shrestha *et al*., 2011) and this may be conserved throughout the family.

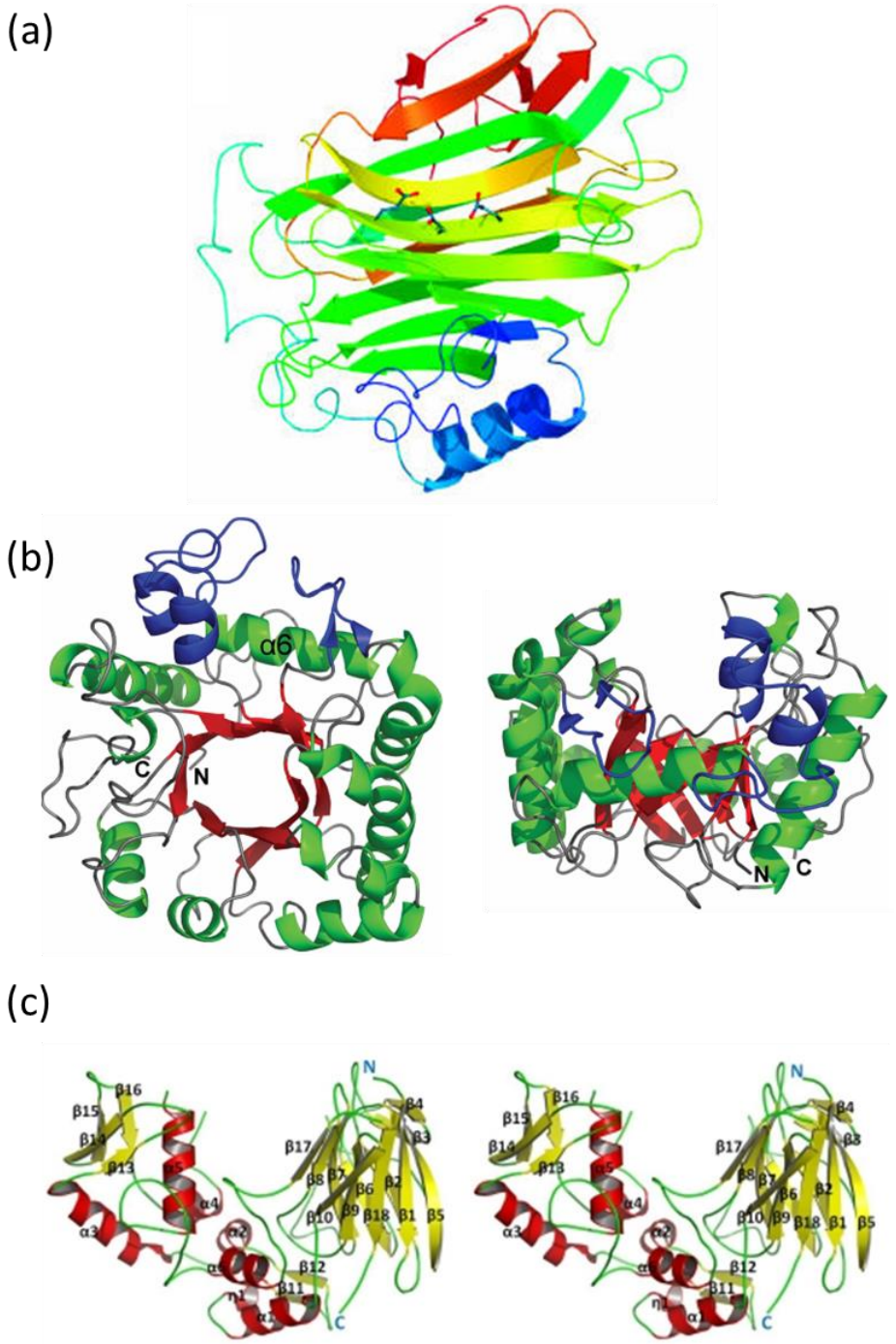


Figure 10: Crystal structures of representative GH16, GH17 and GH64 enzymes

Crystal protein structures of (a) a GH16, PttXET16a (Johansson *et al*., 2004), (b) a GH17, endo-(1,3)-β-glucanase from *S. tuberosum* (Wojtkowiak *et al*., 2011), and (c) a GH64, LPHase (Wu et al., 2009). Figure 10a displays the typical β-jellyroll fold of GH16 enzymes, with active site residues E85, D87 and E89 shown on β7. The protein is colour coded from the N-terminal (red) to the C-terminal (blue). Figure 10b shows top (left) and side (right) views of the GH17 protein with the β-strands of the inner β-barrel encapsulated by α-helices. Additional helices and a β-sheet are shown in blue. Figure 10c shows the GH64 protein with numbered β-strands (yellow) and α-helices (red).

1.7 Project aims and outline

The aim of this project is to survey a broad spectrum of land plants – including bryophytes, gymnosperms, lycopodiophytes, monocots and dicots – for novel transglycanase activities using dicot cell wall matrix polysaccharides including cellulose, hemicelluloses (e.g. mannans, glucans and xylans) and pectins (e.g. galactans and arabinans) as substrates. Potential practical applications of new transglycanase activities are numerous; of particular interest is the possibility of manipulation of cell wall extension, adhesion and other physiological processes to benefit crop plants. As discussed in 1.4.6, the potentials for novel transglycanase activities are vast due to differences in prevalence, substrate specificity, expression stimuli and numerous other variables but detection may be difficult, therefore justifying the completion of a broad-spectrum study, rather than a more focussed one. Increased breadth of plant samples, donors and acceptors makes identification of novel transglycanases more likely. Identification of any novel activity resulting from this survey will result in a more focussed characterisation of the transglycanase activity.

The second focus of this project is the development and optimisation of a heterologous *Pichia pastoris* expression system for the synthesis of recombinant proteins in order to identify the *Equisetum* gene encoding MXE. At present only homotransglycanase genes have been successfully identified and expressed in heterologous expression systems. Identification of a heterotransglycanase gene would be the first of its kind. As such, expression and isolation of a recombinant protein with predominant heterotransglycanase activity would allow further analysis, including enzyme characterisation and structural analysis, to determine if and how it differs from XET and other homotransglycanases.

2 Materials and Methods

2.1 General Materials

Dialysis tubing (12–14-kDa cut-off) came from Medicell International, Ltd. (London, UK). Miracloth was purchased from Calbiochem whilst Whatman chromatography paper (No. 1 and 3) was purchased from VWR (Lutterworth, UK).

Solvents were from Fisher Scientific (Loughborough, UK) and other general chemicals came from Sigma-Aldrich.

2.2 Plant Sources

Snowdrops (*Galanthus nivalis*), crocuses (*Crocus longiflorus*), Yorkshire fog grass (*Holcus lanatus*) and *Equisetum fluviatile* were all collected from the King's Buildings, University of Edinburgh, whilst *Equisetum arvense* was taken from a roadside in the nearby Cameron Toll area. Mung bean (*Vigna radiata*), asparagus (*Asparagus officinalis*), cauliflower (*Brassica oleracea*), chicory (*Cichorium endivia*) and tomato (*Solanum lycopersicum*) were purchased from Sainsbury's (UK). Columbia (Col-0) wild-type *Arabidopsis thaliana* seedlings were kindly contributed by Miss Suzy Howat. Liverwort (*Marchantia polymorpha*) was responsibly sourced from Bonaly Country Park.

2.3 Donor polysaccharide sources

Barley mixed-linkage glucan (β-(1,3)(1,4)-glucan), α-(1,5)-arabinan (branched and linear from sugar beet), RG-I (RG-IA from soy bean pectin and RG-IB from potato pectin), konjac glucomannan (low viscosity), wheat arabinoxylan (medium viscosity), lupin β-galactan (βG2) and borohydride-reduced β-(1,4)-D-mannan were purchased from Megazyme (Bray, Republic of Ireland). Larch wood arabinogalactan (AG Sigma), pectin (homogalacturonan) from orange, galactomannan (guar gum), β-(1,3)-glucan (laminarin) from *Laminaria digitalis*, hydroxyethyl cellulose (HEC) and birchwood xylan were all purchased from Sigma-Aldrich (UK). Tamarind seed xyloglucan was from Dr K. Yamatoya,

Dainippon Pharmaceutical Co. (http://www.ds-pharma.co.jp) whilst 'lab' samples of α-arabinan (αA LN and αA LO) were from the now defunct Koch-Light. Glucuronoxylan, arabinogalactan from larch (AG lab) and β-galactan (βG) were all from cell wall extracts made previously in the lab.

2.3.1 Production of aqueous donor polysaccharide solutions

Donor polysaccharides were added to distilled water (dH_2O), heated to approximately 120°C and mixed using a magnetic stirrer until dissolved to produce a solution of concentration 0.5% (w/v), unless otherwise stated. Donor polysaccharide solutions were stored at room temperature with 0.5% (w/v) chlorobutanol and reheated for 30 minutes in a 100°C water bath prior to use.

2.4 Chromatographic and electrophoretic methods

2.4.1 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

A Bio-Rad Mini PROTEAN Tetra Cell system was used to perform SDS-PAGE. I used the protocol outlined in Laemmli (1970) with gels composed of a 5% resolving gel below a 4% stacking gel. Gels were run at 100 V for ~90 min. The gel was then removed and soaked in fixing solution (40% (v/v) ethanol + 10% (v/v) acetic acid) for 30 min on the shaker and then stained with either silver nitrate (2.5.3) or Coomassie blue (2.5.4).

2.4.2 Paper chromatography

Paper chromatography was used to remove excess unreacted tritiated acceptor oligosaccharide, allowing retention of transglycanase product at the origin for further analysis. Samples were pipetted as streaks at the chromatogram origin, a pencil line 90 mm from one short edge of Whatman 1 or 3MM chromatography paper. The paper was folded along the same edge at 20 mm and 70 mm and run for >48 h in ethyl acetate/ acetic acid/ dH_2O (EAW, 10:5:6) to remove unreacted [^{3}H]XXXGol.

Once dry, a 4 cm x 3 cm section around each sample at the origin was cut from the chromatogram, rolled into a cylinder and placed inside a 22-ml scintillation vial, loaded-side facing outwards. Goldstar Organic liquid scintillation cocktail (Meridian) was added (2 ml) and incubated and assayed for ^{3}H twice for 5 minutes with the scintillation counter.

2.4.3 Thin-layer chromatography

Samples were applied along a pencil line drawn 20 mm from the bottom of an unwashed Merck silica-gel 60 TLC plate (VWR, Lutterworth, UK). Once dry, the TLC plate was run in either butan-1-ol/ acetic acid (glacial)/ dH_2O (BAW, 2:1:1 or 4:1:1) for 7-8 h or ethyl acetate/ pyridine/ acetic acid/ dH_2O (EPyAW, 6:3:1:1) for 3-4 h. For greater definition of bands on the TLC, the plate could be run multiple times in the chosen solvent, with drying between. Detection of digestion products were by thymol staining (see 2.5.1) or fluorography (see 2.5.2).

2.4.4 Size-exclusion chromatography (SEC)

Size-exclusion chromatography (SEC) was carried out using two different Bio-Gel P-2 columns.

Column 1: 14 mm internal diameter, 88 cm^3 column bed volume

Column 2: 10 mm internal diameter, 40cm^3 column bed volume

For each column, the void volume (K_{av} 0) and included volume (K_{av} 1) were determined using 5-40 MDa Blue Dextran and [^{3}H]dH_2O or glucose respectively.

2.4.5 Immobilised metal-ion affinity chromatography (IMAC)

IMAC for purification of His-tagged proteins was performed on a 2-ml bed volume Chelating Sepharose Fast Flow column (Amersham Biosciences, UK) pre-charged with 0.2 M nickel sulphate. All experiments were conducted at 4°C. The binding buffer was 0.02 M sodium phosphate + 0.75 M NaCl (pH 7.0) whilst the elution buffer was further supplemented with 0.05 M imidazole. The column was washed thoroughly with 5-6 column volumes of binding buffer prior to the addition of

crude enzyme solution (*P. pastoris* spent medium in binding buffer; 10 ml) which was passed through the column under gravity. Once the enzyme solution had passed through, the column was washed with a further 2-3 column volumes of binding buffer to ensure all excess unbound *Pichia* spent medium had been removed. The nickel-bound His-tagged proteins were eluted from the column by washing with 10 ml of elution buffer. Fractions of 250 µl were collected.

Fractions from elution of the column were tested for MXE activity (as described in 2.8.7.1) and MXE containing protein fractions were pooled and further purified to remove the imidazole on a Bio-Gel P-2 (40 ml bed volume) column eluted with pyridine/ acetic acid/ dH_2O (PyAW, 1:1:98).

2.5 Staining and quantification methods

2.5.1 Thymol staining of silica-TLCs

Thin-layer chromatograms (TLCs) were stained for detection of polysaccharide digestion products via dipping through 5% (v/v) sulphuric acid, 0.5% (w/v) thymol in ethanol. TLCs were air-dried prior to baking at 105°C for ~ 5 minutes (Jork *et al.*, 1994).

2.5.2 Fluorography

TLCs containing ^3H-labelled compounds were dipped in a fluor solution of 7% (w/v) PPO in (diethyl)ether. Once dry, plates were incubated in a cassette with pre-flashed Kodak BioMax MR film at -80°C.

2.5.3 Silver nitrate staining of Native PAGE gels

Prior to silver nitrate staining, gels were incubated in fixing solution (50% (v/v) ethanol, 12% (v/v) acetic acid, 1.875% (v/v) formaldehyde) for 30 min with constant agitation. Gels were washed thrice in 50% (v/v) ethanol and then once in aqueous sodium thiosulphate (0.01%) for 60 s. To ensure sodium thiosulphate was removed, the gel was washed a further three times in dH_2O prior to staining with silver nitrate (0.1% (w/v) $AgNO_3$, 2.77% (v/v) formaldehyde) for 20 min

with constant agitation. Gels were washed in dH_2O a further 3 times and then developed in a solution of 3% (w/v) sodium carbonate, 18.75% (v/v) formaldehyde and 10^{-4}% (w/v) sodium thiosulphate until bands appeared. To stop staining, the gel was incubated in a solution of 4% (w/v) Tris + 2% (v/v) acetic acid.

2.5.4 Coomassie blue staining of SDS-PAGE gels

SDS-PAGE gels were washed three times for 5 min under constant agitation with dH_2O prior to incubation with GelCode Blue stain (Pierce) for 1 h with shaking. Destaining was then performed via multiple incubations with agitation with destaining solution of 20% (v/v) MeOH, 10% (v/v) acetic acid until the background of the gel was clear.

2.5.5 Bradford assay for total protein concentration

To protein extract (0.8 ml), Bio-Rad Protein Assay Dye (0.2 ml) was added and mixed thoroughly by inversion. A_{595} was measured and a range of known concentrations of bovine serum albumin (BSA) was used for calibration.

2.6 Enzyme and Chemical treatments

2.6.1 Acid hydrolysis

Mild TFA hydrolysis leads to the cleavage of furanose but not more stable pyranose bonds (Kerr & Fry, 2003) within polysaccharides and was carried out via incubation of the polysaccharides with 0.1 M trifluoroacetic acid (TFA) at 85°C for 1 h.

Full-acid hydrolysis was used to hydrolyse polysaccharides to their constituent monosaccharides and was conducted with 2 M TFA at 120°C for 1 h.

2.6.2 Xyloglucan endoglucanase (XEG) digestion of donor polysaccharides

XEG (EC 3.2.1.4) is an *endo*-β-(1,4)-D–glucanase purified from *Aspergillus aculeatus*, a kind gift from Novo Nordisk A/S (Bagsværd, Denmark; Pauly *et al*., 1999b). Samples were incubated in 20% (v/v) PyAW (1:1:98) containing 0.5% (w/v) chlorobutanol and 10^{-3}% XEG at room temperature for 1 h. XEG activity was stopped by boiling for 30 min.

2.6.3 Driselase digestion of donor polysaccharides

Driselase, from *Irpex lacteus*, a basidiomycete fungus (Sigma) was purified according to the method described by Fry (2000). Driselase (1% w/v) in PyAW (98:1:1) was added 1:1 to the polysaccharide and then incubated at 37˚C for 48h with shaking. The reaction was stopped with 50% (v/v) formic acid.

2.6.4 Galactanase digestion of XyG and β-galactans

Endo-(1,4)-β-D-galactanase (EC 3.2.1.181) (440 U ml^{-1} in ammonium sulphate; Megazyme) was incubated with transglycanase reaction products at differing concentrations (44.0 U ml^{-1} (10% v/v), 4.40 U ml^{-1} (1% v/v) and 0.44 U ml^{-1} (0.1% v/v)) and for a range of incubation times (1 min, 10 min, 100 min and 1000 min) to determine the optimum conditions, 1% for 100 min, for the digestion of β-galactan (see 4.3.6).

2.6.5 Cellulase digestion of XyG and β-galactans

Endo-cellulase (EGII), an *endo*-(1,4)-β-D-glucanase (EC 3.2.1.4) from *Trichoderma longibrachiatum* (940 U ml^{-1} in ammonium sulphate, Megazyme) was used to digest XyG from transglycanase reaction products. The optimum conditions, 9.4 U ml^{-1} (1% v/v) for 100 min, were found using the method described in 2.6.4.

2.7 Exploring novel transglycanase activities in the plant call wall (Chapters 3, 4, and 5)

2.7.1 Buffer A production

Buffer A refers to a non-volatile 0.2 M succinic acid (Na^+) buffer produced through adjustment of a 0.2 M succinic acid solution to pH 5.5 using 17.0 M NaOH and addition of $CaCl_2$ (10 mM).

2.7.2 Enzyme extraction procedure

All procedures were conducted at 4°C unless otherwise stated. A pre-weighed mass of finely chopped plant material was homogenised in Buffer A with a hand-held blender in a ratio between 1:2 and 1:5 (g fresh weight: ml Buffer A). The resulting homogenate was incubated for 3 h with agitation, to ensure release of cell wall enzymes into solution, prior to filtration through a double layer of Miracloth and bench centrifugation, using an Eppendorf 5810 centrifuge with A-4-62 rotor, at 3 217 x g for 20 minutes.

2.7.2.1 Ammonium sulphate precipitation for protein extraction

Protein precipitation from homogenised plant samples was carried out with 75% saturated ammonium sulphate incubated overnight at 4°C before being divided into 1-ml aliquots and centrifuged using a MSE Micro Centaur Plus microcentrifuge at 14 573 x g for 15 minutes. The supernatant was pipetted off, discarded and remnants removed with a paper towel. The pellet was then resuspended in 75% saturated ammonium sulphate in buffer A and centrifuged, and the supernatant discarded again. This process was repeated a further 3 times. The pellet was resuspended in buffer A. For enzyme extracts used in section 5, the volume of buffer A used correlated to that required to allow a final extraction ratio of 1:2 (fresh weight (g) : buffer A (ml)). The enzyme extract was stored at -20°C.

2.7.3 Fluorescence assays

2.7.3.1 Synthesis of sulphorhodamine (SR)-labelled acceptor oligosaccharides

2.7.3.1.1 Synthesis of oligosaccharidyl-1-amino-1-deoxyalditols (OADs)

Reductive amination reagent (RAR) was prepared using saturated aqueous ammonium hydrogen carbonate (NH_4HCO_3; added to dH_2O stirred at room temperature) with 640 mM sodium cyanoborohydride ($NaCNBH_3$) added immediately prior to use. Oligosaccharide (1 mg) was dissolved in 25 µl of RAR (containing ~ 0.17 mmol $NaCNBH_3$) and incubated in the dark at room temperature for 8 days.

Following incubation, samples were dried and then resuspended in 10 µl 5% acetic acid. This was repeated 3 times to remove ammonia. Finally the sample was dissolved in 10 µl of dH_2O and dried.

Samples were re-dissolved in 2 ml dH_2O and passed through a Bio-Gel P-2 column (88 ml bed-volume) with dH_2O as the eluant. Sixty fractions of ~1.25 ml were collected and 5 µl of each was loaded onto a 20 x 20 cm silica-gel TLC plate. These plates were subsequently run for 7 hours in BAW (2:1:1) and stained with thymol/ H_2SO_4 once dry (see 2.5.1). A 10 µg solution of the relevant unmodified oligosaccharide was loaded as a marker. Fractions containing the oligosaccharidyl-1-amino-1-deoxyalditol (OAD) (as detected on TLC) were pooled and dried.

2.7.3.1.2 Preparation of a series of oligosaccharide-SR conjugates

Each OAD pool (~0.8 mg) was dissolved in 160 µl dH_2O and mixed with 5 mg solid lissamine rhodamine sulphonyl chrloride (17 µmol) (LRSC; Invitrogen) and 10 mg dry disodium tetraborate (52 µmol). Following overnight incubation, with shaking, each sample was diluted with a further 1.5 ml dH_2O and shaken vigorously to ensure all solid is dissolved. Each sample was passed through a Bio-

Gel P-2 column (88 ml bed-volume) with PyAW (1:1:25). As before, fractions were collected and 5 µl of each was loaded onto silica-TLC plates to identify – via detection with a 254 nm UV lamp – the fractions containing the desired SR-labelled conjugate. These fractions were pooled and dried for further analysis. LRSC+H_2O, LRSC+ammonia and LRSC+glucosamine were loaded as markers (2-10 µg).

2.7.3.1.3 Determining the concentration of OAD pools

Samples of SR-labelled OADs were dissolved in 2 ml dH_2O and the absorbance of a 1-ml aliquot at 567 nm was measured using a spectrophotometer. Distilled water was used as a blank for comparison. From this the concentration of each SR-labelled OAD pool could be determined using the Beer Lambert law.

2.7.3.2 Transglycanase reaction with a fluorescent acceptor oligosaccharide

An aliquot (20 µg) of the appropriate sulphorhodamine-labelled oligosaccharide acceptor (Xyl_6-SR or XXXG-SR) was dried to give zero volume before 5 µl donor polysaccharide was added, centrifuged and mixed. At time zero, 5 µl enzyme extract was added to the reaction mixture and incubated at room temperature for 12 hours. The reaction was stopped by loading samples onto silica TLC-plates.

2.7.3.3 Thin-layer chromatography of transglycanase reaction mixtures

Following overnight incubation of reaction mixtures, 5 µl of each sample (2 x 2.5 µl loaded with drying between aliquots) and 10 µg of Xyl_6-SR and XXXG-SR markers (low Rf) were loaded onto a silica-gel TLC plate which was run in BAW (2:1:1) for 7 hours at room temperature. TLC plates studied with a 254 nm UV lamp. Images were obtained using a MultiDoc-It Digital Imaging System (UVP) and edited using Adobe Photoshop Elements 2.0.

2.7.4 Radio-assay for novel transglycanase activities

Unless otherwise stated, a total reaction mixture of volume 40 µl, containing 30 µl donor polysaccharide (0.5% w/v), 10 µl enzyme extract, 1 kBq [^{3}H]XXXGol

(dried) was incubated for 18 hours at room temperature. The reaction was stopped by addition of 100 µl of 20% (v/v) formic acid. Unreacted [^{3}H]XXXGol was removed by washing via paper chromatography and samples were assayed for radioactivity as described in 2.4.2. Tritium-labelled oligosaccharides were prepared as described in Smith & Fry (1991).

2.8 Cloning of putative XET genes into *Pichia pastoris* (see 6 to 6.9)

All equipment was sterilised and assays conducted within flow-hoods to prevent contamination.

2.8.1 PCR of cDNA

High-fidelity PCR was conducted using specifically designed primers (courtesy of Dr. I. Van Den Brande; Bayer CropScience, Belgium) and Phusion® High-Fidelity DNA Polymerase (New England Biolabs, USA) according to the manufacturer's instructions via the addition of cDNA from *E. fluviatile*. Unless otherwise stated, the PCR conditions used were 30 rounds of denaturing (94°C, 30 s), annealing (58°C, 30 s) and extension (72°C, 30 s).

2.8.2 Infusion PCR cloning

Positive (with cDNA) and negative (without cDNA) PCR products for each construct (10 µl) were run on a 1% agarose gel with 0.5 µg ml^{-1} ethidium bromide for 30 min at 80 V. The expected molecular weight corresponding to each clone was calculated and the appropriate band extracted. Methylated DNA was cut with DPN1 (EC 3.1.21.4; New England Biolabs, USA), degrading the original plasmid and thus allowing the cloned construct DNA to be later incorporated into the pPICZαA plasmid (Invitrogen by Life Technologies, CA, USA).

The reaction product was diluted with TE buffer (pH 8.0) and used for Infusion PCR cloning into the pPICZαA vector as per manufactorers instructions (Invitrogen Life Technologies, 2010). TE buffer contains 10 mM Tris-HCl + 1 mM EDTA.

2.8.2.1 Transformation of Escherichia coli

The Infusion PCR cloning product (5 µl) was used in transformation via thermoporation of 100 µl HC1061 thermocompetent *E. coli* cells (Life Technologies, CA, USA); ice, heat shock at 42°C for 30 s, and back on ice for 2 mins. *E. coli* media (500 µl) was cultured on LB + zeocin (Life Technologies, CA, USA; 4000x) plates and incubated for 1 h at 37°C with shaking. Colonies containing the insert were added to 500 µl SOC media and incubated for 1 h at 37°C with shaking. SOC media refers to Super Optimal Broth (SOB, 2% w/v bacto-tryptone + 0.5% w/v yeast extract + 10 mM NaCl + 2.5 mM KCl in dH_2O) + 10 mM $MgCl_2$ + 20 mM glucose. The plasmid was then transformed into TOP10 electrocompetent *E. coli* cells (Life Technologies, CA, USA) via electroporation and spread onto LB+Km plates (Invitrogen Life Technologies, 2010). The clone was analysed by dot-blot (see 2.8.6) and then sequenced.

2.8.2.2 Transformation of Pichia pastoris

A preculture of 5 ml yeast extract peptone dextrose (YPD; Life Technologies, CA, USA) inoculated with 100 µl SMD1168H *P. pastoris* cell suspension (Life Technologies, CA, USA) was grown overnight at 28°C and then diluted with further YPD to 3 different concentrations. Following overnight incubation, the optical density (OD; A_{600}) of each sample was measured at 600 nm. The optimum sample was selected (A_{600} ~5), centrifuged at 3 217 x g for 20 min and the supernatant discarded. The pellet underwent repeated cycles of resuspension in dH_2O at 4°C (80 ml and then 40 ml), centrifugation and removal of supernatant prior to a cycle of resuspension and centrifugation in 1 M sorbitol (8 ml). Cells were finally resuspended in 300-600 µl sorbitol and stored on ice (Invitrogen Life Technologies, 2010).

Plasmids were linearised via *Pme*I (EC 3.1.21.-; New England Biolabs, USA) digestion for 3 hours at 37°C and then used to transform *P. pastoris* cells via electroporation in 1-mm cuvettes (400 Ω, 1.8 kV, 4-5 ms) using a Biorad Genepulser. Sorbitol (1 ml; 1 M) was immediately added and the cells incubated for 1 hour at 28°C and then again for 1 hour at 28°C with YPD and glucose buffer

before being plated onto YPDS + zeocin (1000 µg µl^{-1}) plates. Cultures were grown for 5 days at 28°C.

2.8.3 Growth and expression media

Various media were tested to determine the optimum for the growth and expression of each construct. The recombinant *P. pastoris* expression system for a GH16 or GH64 insert involved growth and expression in BMGY and BMMY respectively. Expression of GH17 379 was only possible in MM whilst other GH17 recombinant proteins (399 and 400) could only be expressed at extremely low concentrations in BMM. Details of growth and expression media are shown in Table 1.

Table 1: Growth and expression media for XET recombinant proteins from the *Pichia pastoris* heterologous expression system
Yeast nitrogen base (YNB) and Yeast Peptone (YIP) were purchased from Invitrogen and are as described by the Invitrogen Life Technologies manual (2010). P-buffer refers to a 1 M potassium phosphate buffer (pH 6.0). All concentrations are given as v/v unless otherwise stated.

Medium	YNB (10x)	Glycerol (10% w/v)	P-buffer (1 M)	YIP (10x)	MeOH (10% w/v)	dH_2O
BMGY	10%	10%	10%	70%	-	-
BMMY	10%	-	10%	70%	10%	-
MM	10%	-	-	-	10%	80%
BMM	10%	-	10%	-	10%	70%
MMY	10%	-	-	10%	10%	70%

All media were supplemented with biotin (4 x10^{-5}%) whilst zeocin (100 µg ml^{-1}) was also added to the chosen growth medium.

2.8.4 Small-scale expression experiment

Cultures were grown in BMGY overnight (12 clones/ construct) prior to incubation in BMMY for > 4hours (Invitrogen Life Technologies, 2010). Unless

otherwise stated, incubations were conducted at 28°C. The harvested supernatant underwent dot-blot analysis (see 2.8.6). From this the optimum *Pichia* clone for a large scale expression assay for each construct was deduced. Strains were stored at -80°C in 1 ml YPD + 200 µl glycerol.

2.8.5 Large-scale expression experiment

The optimal *Pichia* clone for each construct was inoculated in 5 ml YPD + 200 µl 80% glycerol and grown overnight to produce a preculture of which 1 ml was added to 250 ml BMGY. Following overnight incubation, the medium was exchanged for a sufficient volume of BMMY to ensure an optical density ~1. Expression proceeded for > 4 hours.

Following expression, samples were centrifuged at 3 217 x g for 20 min at 4°C. The supernatant, containing the desired *Pichia*-secreted protein, was stored at 4°C while the pellet was discarded. Although the unconcentrated *Pichia*-expression product was often used for further analysis, it was possible to microfilter and further concentrate the desired protein (and other native *Pichia* secreted proteins). In this case, the supernatant underwent multiple centrifugations at 3 217 x g for 30 min at 4°C using Amicon® UltraCel®-10K Regenerated Cellulose (MW cut-off = 10 000) centrifugal filters (Merck Millipore Ltd., Ireland). The supernatant retained on the filter was pipetted off and stored at -20°C until further use.

2.8.6 Dot-blot analysis

For dot-blot analysis the following buffers were used: Towbin buffer (TB) refers to 25 mM Tris + 192 mM glycine + 20% methanol at pH 8.3, TBST (pH 7.4) contains 50 mM Tris-buffered saline, 100 mM NaCl and 0.05% Tween 20 whilst buffer 2 is 100 mM maleic acid + 150 mM NaCl + 1% blocking reagent (milk powder).

BMMY-incubated cultures for the 12 colonies for each construct were centrifuged at 3 217 x g for 30 minutes. Supernatant (100 µl) of each sample was spotted onto

nitrocellulose paper, pre-wetted with TB, followed by a washing of 100 µl TB buffer and was then dried. The blot was blocked with a minimal volume of buffer 2 and incubated with shaking at room temperature for 1 h. Next the blot was incubated with rabbit anti-*myc* antibody (1/ 5000) (ab9106, Abcam) diluted in TBST + 10% buffer 2 for 2 h at room temperature. This was washed 5 times with dH_2O and then 2 times with TBST for 5 min. The blot was incubated with goat anti-rabbit-HRP (1/ 5000) (ab97051, Abcam) diluted in TBST + 10% buffer 2 for 1 h at room temperature and washed as before. Chemiluminesence was used for detection.

2.8.7 Activity assays

2.8.7.1 XET and MXE activity assay

Unless otherwise stated: XET activity was assayed using a reaction mixture consisting 10 µl *Pichia*-secreted enzyme extract, 1 kBq [^{3}H]XXXGol (dried to give zero volume) and 10 µl donor xyloglucan (XyG) polysaccharide (1%). Donor, enzyme and acceptor were in 50 mM MES buffer (pH 6.0). The reaction mixture was incubated for 16 h at room temperature and the reaction stopped by addition of 50 µl of 50% (v/v) formic acid. Each sample was loaded onto Whatman 3MM filter paper, dried and then washed thoroughly with free-flowing water* to remove unreacted [^{3}H]XXXGol. Each sample was dried, incubated with Goldstar Organic Liquid Scintillation Cocktail (2 ml) and assayed for radioactivity twice for 5 minutes. “Enzyme-free” controls involved the addition of formic acid prior to the addition of enzyme to produce an environment in which it is unable to function.

The MXE activity assay differs from the XET assay by the use of MLG (1%) as the donor polysaccharide instead of XyG.

*Time taken for removal of excess radioactivity was determined by assaying square of paper on which 1 kBq [^{3}H]XXXGol alone loaded. It was washed in the

same conditions as those containing the complete reaction mixture, producing levels of radioactivity equivalent to background.

2.8.8 CXE activity assay

Unless otherwise stated: To 1 kBq dried [^{3}H]XXXGol, 33 µl enzyme extract (in 50 mM MES; pH 6.0) was mixed thoroughly and added to 10 mg of pre-treated (see 2.8.8.1) dry Whatman No. 1 paper and incubated at room temperature for 16 h. The reaction was stopped by the addition of 300 µl 10% (v/v) formic acid before repeated washing for 8-16 hours to remove unreacted [^{3}H]XXXGol. Following the final washing and removal of excess water, cellulose was resuspended in 0.2 ml dH_2O and 2 ml ScintSafe 3 liquid scintillant cocktail (Fisher Scientific, UK). This was poured into a scintillation vial, repeated and incubated for 24 hours prior to assaying for radioactivity.

2.8.8.1 NaOH pre-treatment of Whatman No. 1 chromatography paper

Whatman No. 1 paper was incubated overnight at 37°C in 45 ml 6.0 M NaOH. The paper was then subjected to numerous washing steps with water, until the washings had a pH of 7.2, before being washed with a solution of PyAW (33:1:300, pH 6.5). The paper was then washed again until the washings has a pH ~6.8. Finally, the paper was lyophilised, and aliquoted by mass.

2.8.9 Further analysis

2.8.9.1 Dephosphorylation

Acid phosphatase from *E. coli* (17 U mg^{-1}; Megazyme), an orthophosphoric-monoester phosphohydrolase (EC 3.1.3.2), was used in the dephosphorylation of recombinant proteins to determine the importance of phosphorylation as a post-translational modification for XET/ MXE function. A reaction mixture of 1 kBq [^{3}H]XXLGol (dried to give zero volume), 10 µl donor polysaccharide (3 mg ml^{-1} XyG or 7.5 mg ml^{-1} MLG) in 50 mM MES buffer (pH 6.0), 10 µl enzyme extract (*Pichia* spent medium or beansprout) was incubated for 16 h and the reaction

stopped with 20 µl 50% (v/v) formic acid. The samples were then dried prior to addition of 40 µl acid phosphatase diluted 20-fold in sodium acetate buffer + 1.0 mg ml^{-1} BSA + 2 mM $MgCl_2$ + 0.2 mM $ZnSO_4$ and tested for radioactivity incorporated at 2-h intervals up to 24 h.

2.8.9.2 De-glycosylation

The effect of de-glycosylation on the XET/ MXE activity of the recombinant proteins was tested using EndoH (New England Biolabs, USA). EndoH refers to Endoglycosidase H (EC 3.2.1.96), a recombinant glycosidase which cleaves high mannose *N*-glycans from glycoproteins. Deglycosylation was conducted using the same method described for dephosphorylation (2.8.9.1) but rather than acid phosphatase, reaction mixtures were resuspended in 40 µl EndoH (5 U $µl^{-1}$) in 50 mM sodium citrate buffer (pH 5.5).

2.8.9.3 Viscosity assays

To determine whether native *Pichia* secreted proteins degraded MLG, viscosity assays were conducted. In 50 mM MES (pH 6.0) 0.4% MLG was mixed in a ratio of 10:1 (MLG : native *Pichia* protein). The native *Pichia* secreted protein was sourced from *P. pastoris* expressing an empty pPICZαA plamid. A control was used replacing native *Pichia* protein with 50 mM MES buffer (pH 6.0). MLG was incubated for 12 h with the *Pichia* extract (or control).

Post-incubation, the MLG was drawn into a 1-ml glass pipette and the time taken for the meniscus to drop 200 µl was measured. The pipette tip was submerged in MLG solution to prevent bubble formation.

2.9 Recombinant MXE expression from *Pichia pastoris* (see 6.10)

Work conducted by Dr. Tom Simmons led to the extraction of RNA from late-season *E. fluviatile* and subsequent production of a transcriptome sequenced using 454 sequencing technology (Rocke). Dr Simmons also designed the primers and conducted the high-fidelity PCR, site-directed mutagenesis – by the use of the QuikChange method (Quikchange, UK) to create the consensus sequence – and

gene cloning into the pPICZαA:insert vector into *E. coli* (DH5α) using the pJET1.2 vector system (Fermentas) prior to collaborative work to transform *P. pastoris* and express the gene.

The primers used were as follows:

UpstreamFw MXE:pPICZαA (*Eco*RI-incorporating): GAA TCC GGT TTC TAT GGG GAC TTT CAG

DownstreamRv MXE:pPICZαA (*Xba*I-incorporating): TCT AGA TAG AAA CCA CGG TTT GAG CAT T

2.9.1.1 Production of recombinant MXE protein in the Pichia pastoris expression system

Extraction of plasmids (pPICZαA:insert) from positive *E. coli* colonies was conducted by the use of QuiaQuik kit (Quiagen). The plasmid was linearised by *Sac*I (EC 3.1.21.4) and incubated for 1 h in 10% (w/v) 3 M sodium acetate (pH 5.2) and 2.5 volumes of ethanol at -20°C before centrifugation. The supernatant was discarded and the pellet washed with 70% (v/v) ethanol. The pellet was redissolved in dH_2O.

Plasmids (0.2-2 µg) were used to transform pre-washed *P. pastoris* SMD1168H cells (using the method described in the pPICZαA manual, Invitrogen). Electroporation was performed as described in 2.8.2.2 and cells were streaked onto plates (1% (w/v) tryptone, 0.5% (w/v) NaCl, 0.5% (w/v) yeast extract, 1.5 % (w/v) agar, 2% glucose, 100 µg/ ml zeocin; adjusted to pH 7.5 with NaOH). The recombinant protein expression system growth and expression were as described in 2.8.4 except in the growth medium BMGY, YIP was substituted with low salt LB (1% tryptone, 0.5% NaCl, 0.5% yeast extract; adjusted to pH 7.5 with NaOH), and the expression medium was low salt LB + MeOH (1%).

3 Results: Initial broad-spectrum transglycanase assays encompassing both qualitative and quantitative approaches

3.1 Fluorescent assay for novel enzyme activity using sulphorhodamine (SR)-labelled oligosaccharides

XET activity, a positive control, was detected in the enzyme extracts, from homogenised plant material, of all species tested. Enzyme extracts were assayed for XET activity in the presence of donor xyloglucan (tamarind; Tx) and XXXG-SR acceptor. In addition, the fluorescence assay indicated potential novel enzyme activity for a number of different donor : acceptor reactions in all samples (see Section 3.1.1). Transglycanase activity was detected by the presence of a fluorescent band at the origin of the TLC plate indicating successful enzymatic transfer of a section of a large polysaccharide donor to a SR-labelled acceptor oligosaccharide to produce a SR-labelled polymer. Unreacted SR-oligosaccharides were significantly smaller than the reacted SR-polymers and therefore migrated. Unreacted polysaccharide donor did not fluoresce.

3.1.1 XXXG-SR and Xyl_6-SR acceptor substrates indicate novel enzyme activity

All extracts showed a degree of hydrolysis of SR-labelled oligosaccharide. XXXG-SR underwent pair-wise hydrolysis (hepta- > penta- > tri-saccharide) (Figure 11) due to removal of the terminal xylose by α-xylosidase to form GXXG and rapid removal of the terminal glucose by β-glucosidase (Franková & Fry, 2011). By contrast, Xyl_6-SR showed hydrolysis products in a homologous series from hexa- to di-saccharide. It is probable that the crude enzyme extracts used in this assay contained other enzymes capable of hydrolysing the acceptor molecule. The enzyme-free control also showed hydrolysis of SR-labelled oligosaccharides suggesting they are partially unstable but this was consistent with that observed in the fluorescent markers so can be considered negligible (Figure 11f).

Potential novel enzyme activity was detected in beansprout with glucomannan as the donor for both Xyl_6-SR (glucomannan : Xyl_6-SR) and XXXG-SR acceptors. Origin fluorescence is slightly elevated compared to that of the donor-free and enzyme-free controls and correlates with a reduction in hydrolysis of acceptor oligosaccharides (Figure 11a), which could result, for example, from transglycosylation being favoured over hydrolysis in an enzyme with dual-functionality. The same is true of arabinoxylan and MLG with XXXG-SR in cauliflower. In the case of MLG, hydrolysis does not exceed penta-saccharide formation (Figure 11b). Beansprout also showed low level fluorescence with glucuronoxylan and α-arabinan : XXXG-SR and a slight reduction in hydrolysis of the donor polysaccharides.

As expected, all *Equisetum* samples displayed MXE activity with an MLG donor and XXXG-SR acceptor. *E. arvense* was found to have possible enzyme activity in the presence of arabinoxylan : XXXG-SR and glucuronoxylan : Xyl_6-SR shown by high fluorescence at the origin and differences in hydrolysis patterns. There was also an indication of low level activity with glucomannan for both Xyl_6-SR and XXXG-SR (Figure 11c). *E. fluviatile* also indicates enzyme activity for arabinoxylan and glucomannan : XXXG-SR but not for glucuronoxylan : Xyl_6-SR. Hydrolysis patterns of the oligo-SR for α-arabinan : Xyl_6-SR and all XXXG-SR samples differed from that of the controls with the former showing greater hydrolysis and the latter significantly lower (Figure 11d).

Holcus lanatus presented potential novel activity for glucomannan : Xyl_6-SR, detected by low level fluorescence at the origin. This was also true of arabinoxylan : Xyl_6-SR and arabinoxylan : XXXG-SR. Glucomannan : Xyl_6-SR which displayed a higher degree of hydrolysis of hexa- and pentasaccharides to disaccharide whilst arabinoxylan samples presented lower levels of hydrolysis. β-Galactan : Xyl_6-SR displays origin fluorescence higher than that of the control but disappointingly the β-galactan : XXXG-SR was loaded incorrectly. There was an indication of low level fluorescence for α-arabinan, HEC and glucuronoxylan : XXXG-SR which could prove significant in further analysis (Figure 11e).

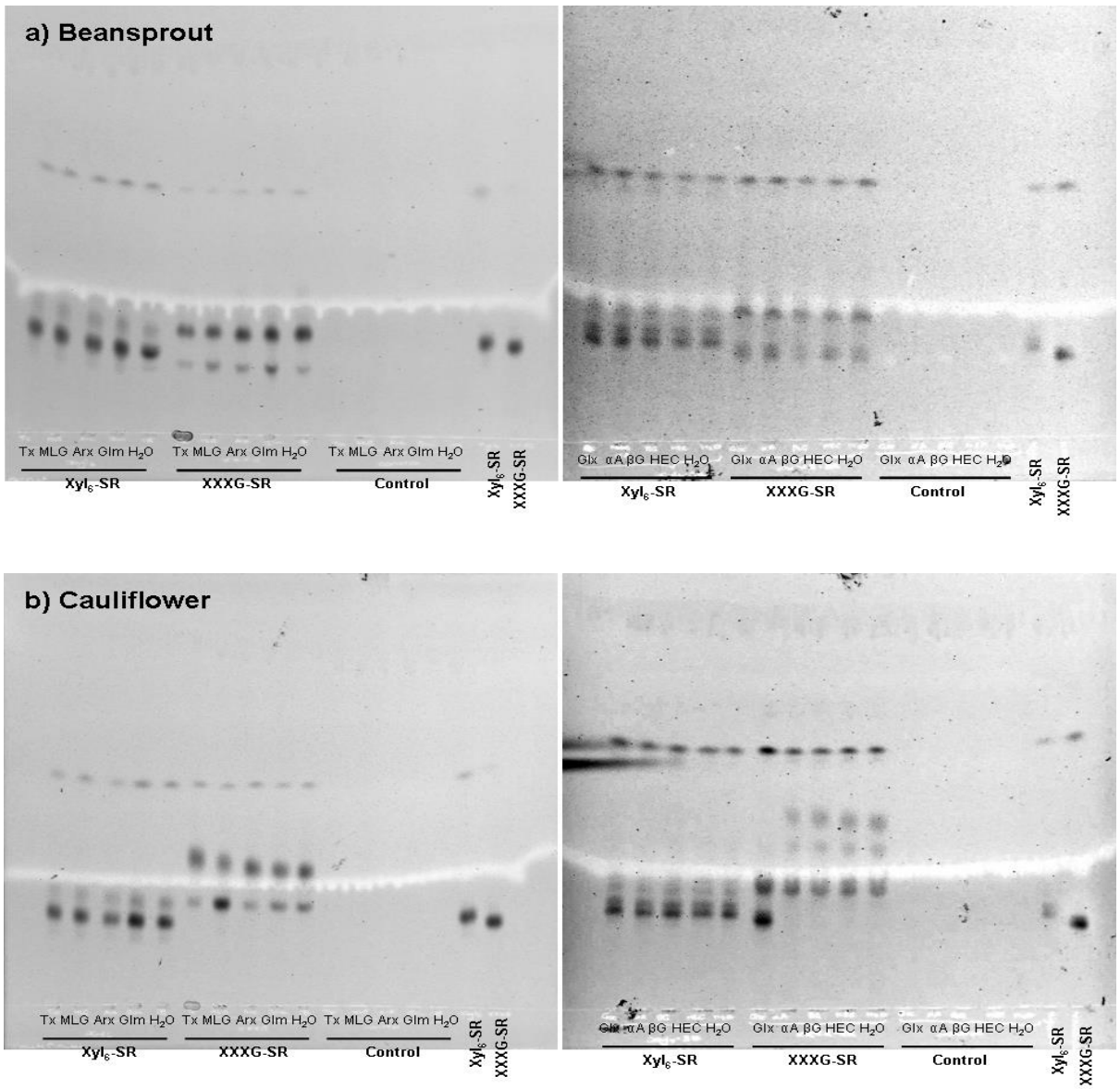
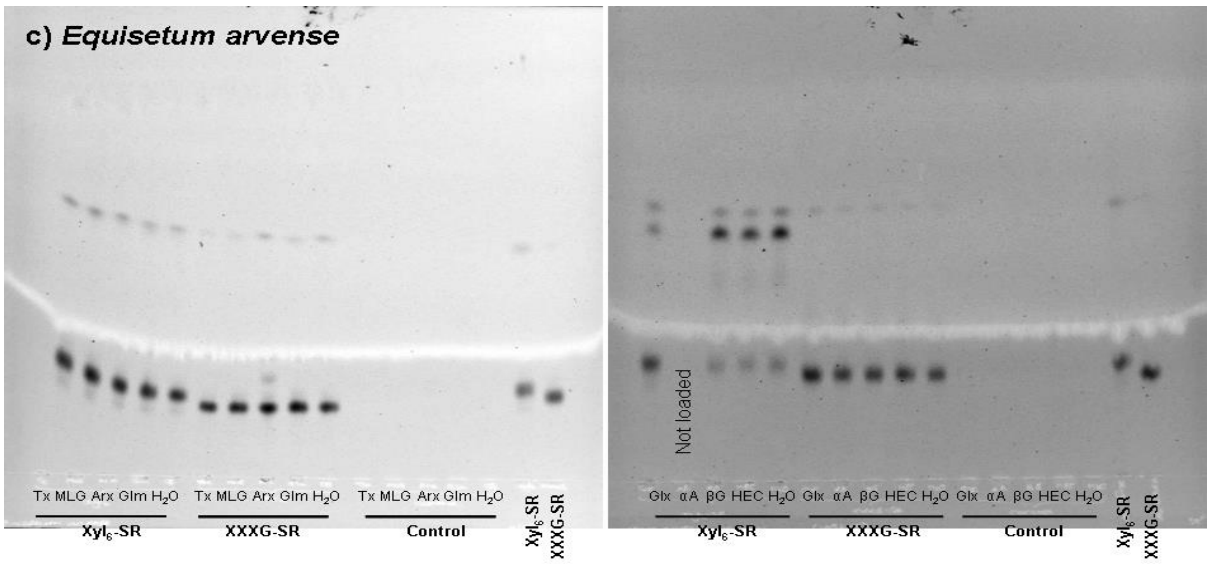
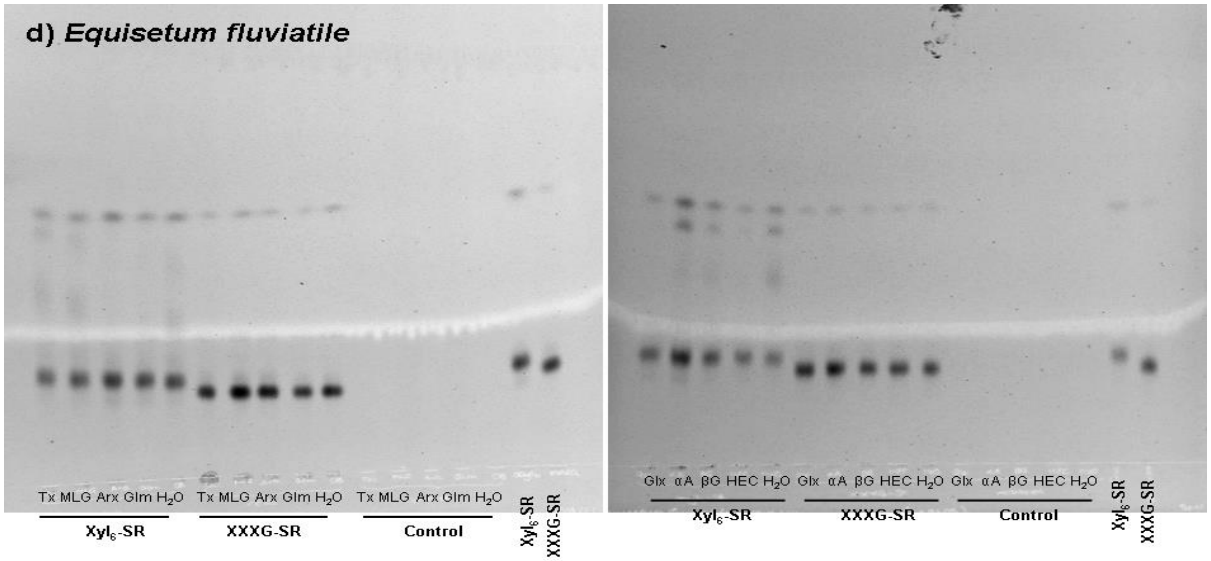
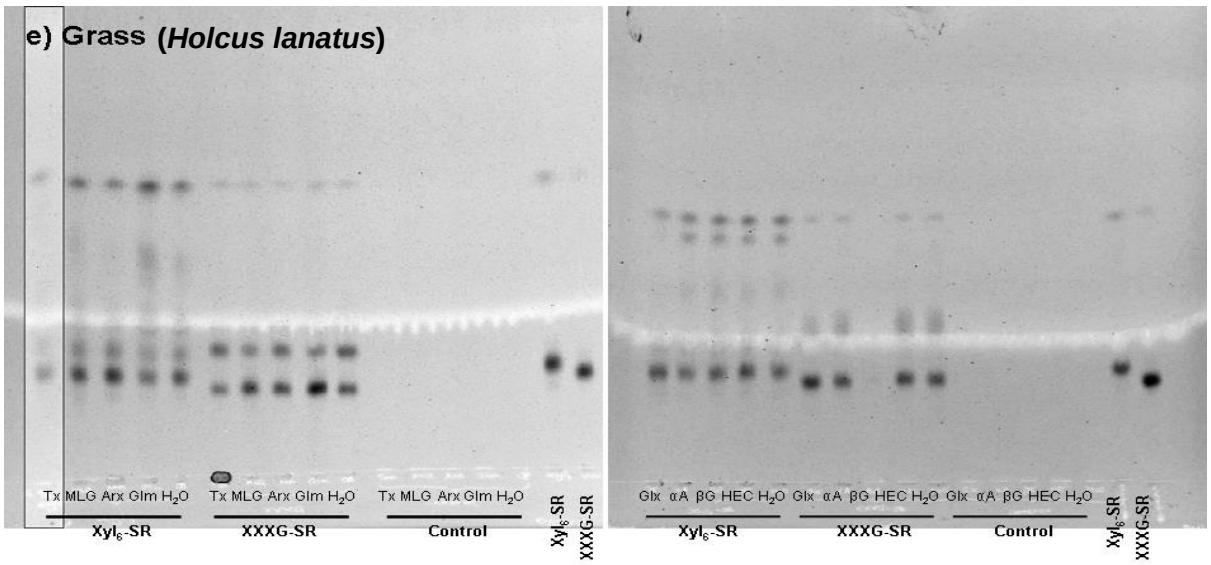


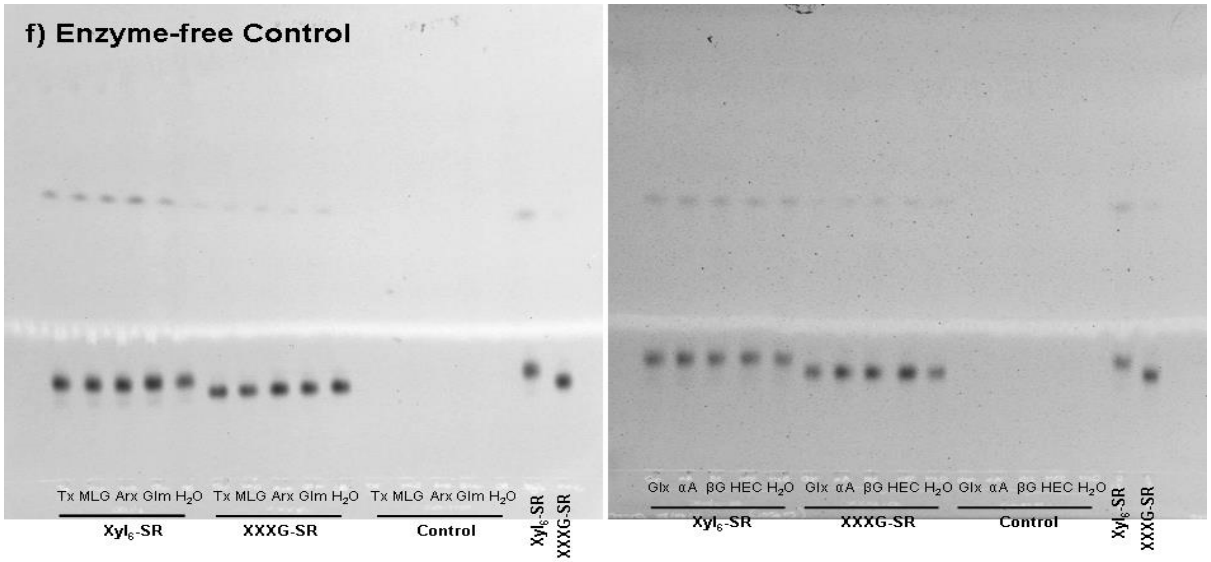
Figure 11: TLCs of a broad-spectrum fluorescent transglycanase assay using a SR-labelled acceptor oligosaccharide
TLC plates showing results for a fluorescent transglycanase assay of enzyme extracts from (a) beansprout, (b) cauliflower, (c) *E. arvense*, (d) *E. fluviatile*, (e) Grass (*H. lanatus*) and (f) enzyme-free control with XXXG-SR, Xyl_6-SR and SR-free control acceptors as outlined in 2.7.3.

Abbreviations: **αA** = α-(1→5)-arabinan (Lab Old), **βG** = β-(1→4)-galactan, **Arx** = Arabinoxylan, **Glm** = Glucomannan, **Glx** = Glucuronoxylan, **HEC** = Hydroxyethyl cellulose, **MLG** = Mixed linkage β-(1→3), (1→4)-glucan, **Tx** = Tamarind xyloglucan


c) Equisetum arvense
Tx MLG Arx Glm H2O
Tx MLG Arx Glm H2O
Tx MLG Arx Glm H2O
Xyl6-SR
XXXG-SR
Control
Xyl6-SR
XXXG-SR
Not loaded
Glx αA βG HEC H2O
Glx αA βG HEC H2O
Glx αA βG HEC H2O
Xyl6-SR
XXXG-SR
Control
Xyl6-SR
XXXG-SR


d) Equisetum fluviatile
Tx MLG Arx Glm H2O
Tx MLG Arx Glm H2O
Tx MLG Arx Glm H2O
Xyl6-SR
XXXG-SR
Control
Xyl6-SR
XXXG-SR
Glx αA βG HEC H2O
Glx αA βG HEC H2O
Glx αA βG HEC H2O
Xyl6-SR
XXXG-SR
Control
Xyl6-SR
XXXG-SR


e) Grass (*Holcus lanatus*)
Tx MLG Arx Glm H_2O Tx MLG Arx Glm H_2O Tx MLG Arx Glm H_2O
Xyl_6-SR XXXG-SR Control
Glx αA βG HEC H_2O Glx αA βG HEC H_2O Glx αA βG HEC H_2O
Xyl_6-SR XXXG-SR Control
Xyl_6-SR
XXXG-SR


f) Enzyme-free Control
Tx MLG Arx Glm H_2O Tx MLG Arx Glm H_2O Tx MLG Arx Glm H_2O
Xyl_6-SR XXXG-SR Control
Glx αA βG HEC H_2O Glx αA βG HEC H_2O Glx αA βG HEC H_2O
Xyl_6-SR XXXG-SR Control
Xyl_6-SR
XXXG-SR

This broad spectrum assay for novel transglycanase activity using XXXG-SR and Xyl_6-SR acceptors has yielded some promising results. The positive result for XET activity for all samples shows that the use of SR-labelled acceptors is viable for presenting evidence of transglycanase activity.

3.1.2 Newly synthesised Man_6-SR shows little activity as an acceptor substrate

A previously untested Man_6-SR oligosaccharide was synthesised by use of the method described by Fry (1997) to investigate the potential for other, non-xylose based, oligosaccharides as suitable acceptor substrates. MET activity wth a Man_6-SR acceptor and a glucomannan acceptor was used as a positive control. Beansprout showed possible fluorescent transglycanase products for glucomannan and α-arabinan : Man_6-SR compared to the negative control. This correlated with a reduced hydrolysis from tetra- to trisaccharide compared to other samples. MLG was accidentally double-loaded so convincing comparison to the control for this donor polysaccharide is not possible (Figure 12a). *E. arvense* showed reduced hydrolysis of hexasaccharide for glucomannan, α-arabinan and glucuronoxylan with low level fluorescence at the origin (Figure 12b). There was no visable production of fluorescent polymers following incubation of potential donor polysaccharides with the *H. lanatus* extract, indicating a lack of transglycanase activity with a Man_6-SR acceptor (Figure 12c).

Although using fluorescently labelled oligosaccharide acceptors to identify novel enzyme activity has been useful in many ways, such as identifying potential new transglycanase activities to focus on further, it has its share of caveats. Whilst some results show a clear fluorescence at the origin, it is difficult to detect lower-level activity. Therefore, the significance of results can be hard to determine. Quantitative analysis is required to determine the credibility of the results obtained from these experiments. A summary of all fluorescent transglycanase assays are included in Table 2.

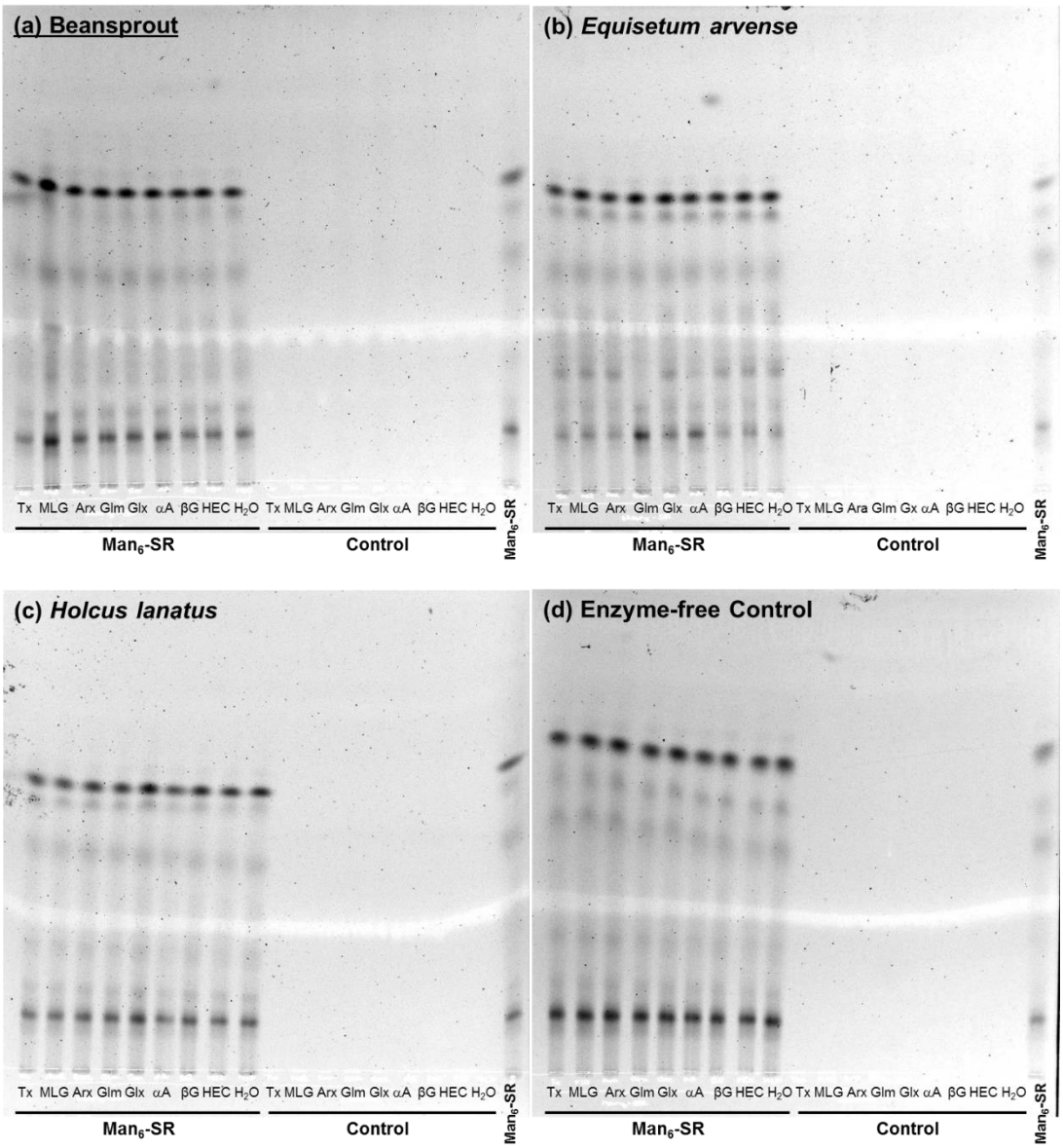


Figure 12: TLCs of a broad-spectrum fluorescent transglycanase assay using a Man_6-SR acceptor oligosaccharide

TLCs of transglycanase assay (2.7.3) products with a range of potential donor polysaccharides (see Figure 11 legend for abbreviations), Man_6-SR acceptor oligosaccharide and enzyme extracts from (a) beansprout, (b) *E. arvense*, (c) grass (*H. lanatus*) and d) enzyme-free control. A SR-free acceptor was used as an acceptor-free control to detect background fluorescence.

3.2 Radioactively Assaying Enzyme Extracts for Activity with different donor polysaccharides

3.2.1 Radio-assay to test for known XET activity in enzyme extracts

All enzyme extracts were assayed for XET activity with a [^{3}H]XXXGol radio-labelled acceptor and xyloglucan donor. All extracts displayed some level of XET activity suggesting extracts prepared previously were enzymatically active (Figure 13). Beansprout displayed the highest levels of XET activity with 663 cpm of radioactivity incorporated following a 12 h incubation which confirms results previously observed during the fluorescent assays. Cauliflower (310 cpm), *Holcus lanatus* (85 cpm) and *Equisetum* (*E. arvense*= 32 cpm, *E. fluviatile*= 55 cpm) samples had significantly lower activity than beansprout but were still observed as above that of the enzyme-free control (11 cpm). These results highlight the suitability of this method for quantifying transglycanase activity and its potential for detecting novel enzymes.

Levels of XET activity are known to differ between species (Fry *et al*., 1992; Campbell & Braam, 1999a) and are also likely influenced by different ratios of dry weight (g) : buffer A (ml) during the enzyme extraction procedure. Future enzyme extracts were concentrated following ammonium sulphate precipitation (2.7.2.1) to ensure a more normalised extraction ratio within the range of 1:1 to 1:2.

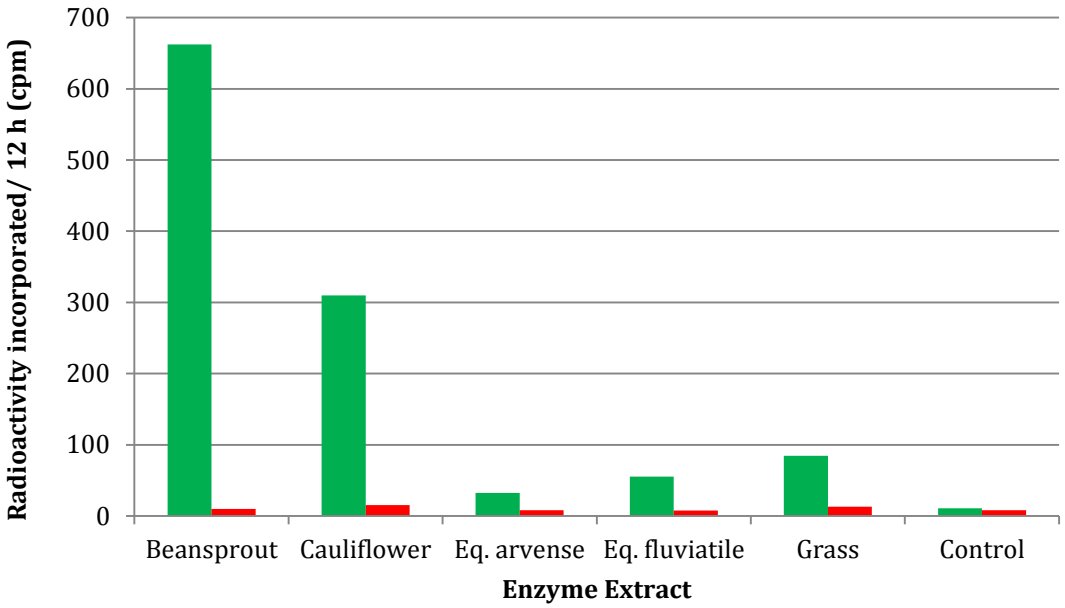


Figure 13: Radioactivity incorporated per 12 h incubation (cpm) for enzyme extracts to assay for the presence of XET activity, indicating an enzymatically active sample
Reactions between a xyloglucan donor + [^{3}H]XXXGol acceptor (green) and donor control (dH_2O) + [^{3}H]XXXGol (red) are shown. 'Grass' refers to the *H. lanatus* enzyme extract. Results are cancelled for a combined donor (H_2O) and acceptor (blank) control.

3.2.2 Radioactive assay for novel transglycanase activities

The assay described in 2.7.4 was modified to include a wider range of donor polysaccharides: arabinoxylan, glucomannan, glucuronoxylan, α-arabinan, β-galactan and hydroxyethyl cellulose. These data indicated a high activity for α-arabinan : [^{3}H]XXXGol in beansprout with radioactivity incorporated (1931 cpm) approximately 10 times that of the control. Glucuronoxylan, β-galactan and HEC all indicated radioactivity incorporated that was significantly higher than that of the control. All control samples showed radioactivity around background levels. *Holcus lanatus* showed significant incorporation of radioactivity with α-arabinan, β-galactan and HEC donors (Figure 14a). The assay was repeated for these samples and different washing methods tested to ascertain optimum conditions (Figure 14b).

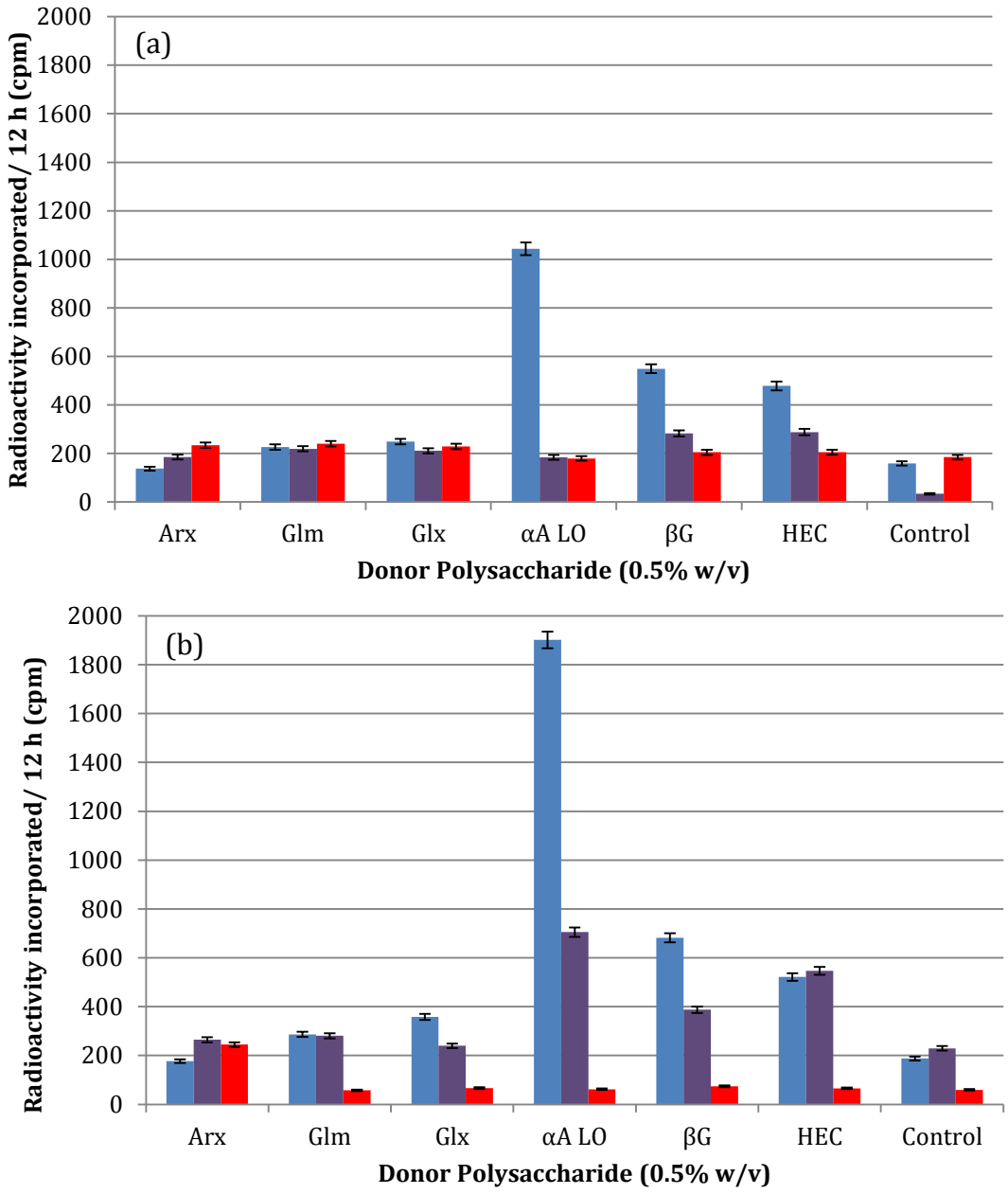


Figure 14: Radio-assay for novel transglycanase activity with excess tritiated-acceptor removed by different washing methods

(a) Primary results using a [^{3}H]XXXGol acceptor and range of donor polysaccharides for beansprout (**blue**), *H. lanatus* (**purple**) and enzyme-free (**red**) extracts. Unreacted [^{3}H]XXXGol was removed by repeated washings with 60% ethanol. Error bars indicate standard error for assays conducted in triplicate.

(b) Results from assays, conducted in triplicate, of beansprout (**blue**), *H. lanatus* (**purple**) and enzyme-free (**red**) extracts with a [^{3}H]XXXGol acceptor and range of polysaccharide donors. As before, a significant peak is observed at α-arabinan for beansprout. All cpm values for the enzyme-free control are lower than that of the primary results, presumably associated with more effective removal of unreacted [^{3}H]XXXGol via paper chromatography in EAW (10:5:6) for 32 hours.

Results indicate potential novel enzyme activities in beansprout with α-arabinan, β-galactan and HEC donor polysaccharides. Of particular significance α-arabinan : [^{3}H]XXXGol resulted in the amount of radioactivity incorporated (1931 cpm and 1054 cpm for washing with 60% ethanol and by paper chromatography respectively) which was approximately 5 to 10 times that of the donor-free control (Figure 14). This is supported by fluorescence results (Figure 11a) and suggests a potential α-arabinan: xyloglucan transglycanase activity (AXE).

Some inconsistencies were observed between primary and repeat results, likely to be due to differing methods used to remove unreacted [^{3}H]XXXGol. Both indicated possible transglycanase activity in grass with β-galactan and HEC donors but there were significant differences in the results between the two experiments meaning that further analysis is required. Enzyme activity for HEC : XGO correlates with fluorescence results but unfortunately there are no fluorescence results for *Holcus lanatus* for β-galactan: XGO for comparison. No origin fluorescence was detectable for beansprout samples with any acceptor and a β-galactan donor, meaning this potential transglycanase activity was not detectable by this method.

Little significant novel transglycanase activity was detected with cauliflower, *E. arvense* and *E. fluviatile* although all extracts showed an increased amount of radioactivity incorporated compared to that of the donor-free control in the presence all potential donor polysaccharides. The amount of radioactivity incorporated remained relatively consistent for all donor polysaccharides for the same enzyme extract indicating that this is more likely an artefact of the assay than detection of novel transglycanase activities (Figure 15).

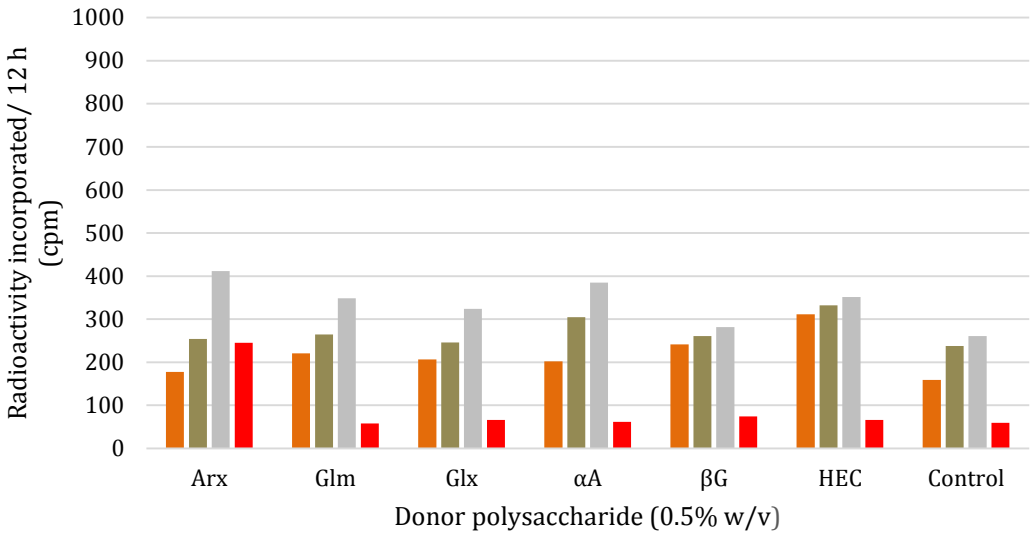


Figure 15: Radio-assay for novel transglycanase activity in cauliflower, *E. arvense* and *E. fluviatile extracts* following 12 h incubation
Results from an unreplicated assay using a [^{3}H]XXXGol acceptor (1 kBq) and a range of donor polysaccharides for cauliflower (orange), *E. arvense* (**gold**), *E. fluviatile* (grey) and enzyme-free (**red**) extracts. Unreacted [^{3}H]XXXGol was removed by repeated washings with 60% ethanol.

Table 2: Summary of fluorescent and radioactive transglycanase assays
Results from all fluorescent (Figure 11 and Figure 12) and radioactive (Figure 13 and Figure 14) transglycanase assays indicating potential novel transglycanase activities. The indicator of potential transglycanase activity observed is described by the following:

+ = origin fluorescence/ incorporation of radioactivity above that of the control

+ = minimal origin fluorescence

H = differing hydrolysis pattern only

- = no radioactivity incorporated significantly above that of the control

Results from the radioassay are indicated for separate washing methods. For example, -/+ indicates that significant activity was observed following removal of excess [^{3}H]XXXGol via paper chromatography (Figure 14b) but not following washing with 60% ethanol (Figure 14a).

XXXG-SR

	Tx	MLG	Arx	Glm	Glx	αA	βG	HEC
BS	+			+	+	+		
Ca	+					H		
EA	+	+	+	+		H		
EF	+	+	+	+		H		
Gr	+		+		+	+	+	+

Xyl_6-SR

	Tx	MLG	Arx	Glm	Glx	αA	βG	HEC
BS				+				
Ca								
EA				+	+			
EF						H		
Gr			+	+			+	

Man_6-SR

	Tx	MLG	Arx	Glm	Glx	αA	βG	HEC
BS				+		+		
EF				+	+	+		
Gr								

[^{3}H]XXXGol

	Tx	MLG	Arx	Glm	Glx	αA	βG	HEC
BS	+		-/+	+/+	+/+	+/+	+/+	+/+
Gr	+		-/+			+/+	+/+	+/+

4 Results: Investigating potential novel AXE and GXE activity

4.1 Increased range of donor polysaccharides

The presence of a transglycosylation activity, with either α-arabinan or β-galactan as the donor polysaccharide for a xyloglucan oligosaccharide acceptor, could provide a mechanism by which observed covalent bonds between pectic and hemicellulosic polysaccharides in the plant cell wall form. To investigate the hypothesis that a transglycanase could be involved in the integration of the hemicellulosic and pectic networks, other pectic donor polysaccharides – an increased range of α-arabinan donors, RG-I from soy bean pectin (RG-IA) and potato pectin (RG-IB) and arabinogalactan (AG Lab and AG Sigma) from larch wood – were tested as donor polysaccharides in a transglycosylation assay with the highly XET-active beansprout extract.

As expected, XET activity products of the beansprout extract were high, reaching an incorporation of radioactivity of 5940 cpm (maximum incorporation for 1 kBq = ~ 15 000) after 16 h incubation, indicating this extract was enzymatically active. Different α-arabinan donors showed significantly different levels of radioactivity incorporated, implying varying levels of transglycanase reaction product. Whilst both the 'new' and 'old' Koch Lite α-arabinan samples (αA LN and αA LO respectively) showed significant product formation (1888 cpm and 3030 cpm respectively), Megazyme branched α-arabinan (αA MB) and Megazyme linear α-arabinan (αA ML) gave counts of just 315 cpm and 366 cpm respectively (Figure 16). This difference in activity could have resulted from a number of factors including polysaccharide chain length, differing branching patterns or possible contamination with other polysaccharides thus giving a false positive for AXE activity. Therefore, further investigation was required.

The other pectic polysaccharide donors – RG-I and arabinogalactan – supported no significant enzyme activity. This could indicate that any potential AXE activity does not occur between the hemicellulosic and pectic networks. However, as the α-arabinan chains of RG-I are relatively short (especially when compared to the

full length polysaccharide of the 'pure' α-arabinan donors) it is plausible that any transglycoylation product could be of insufficient molecular weight to form a ^{3}H-polymer product capable of remaining bound at the origin during removal of excess unreacted [^{3}H]XXXGol following washing via paper chromatography.

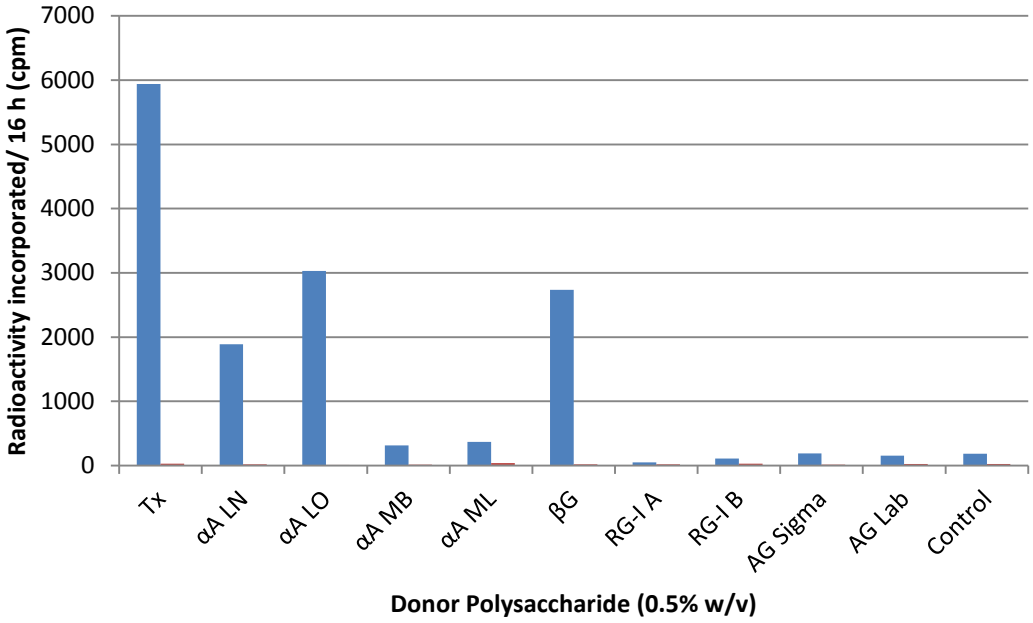


Figure 16: Transglycosylation assay for extended range of pectic potential donor polysaccharides
Selection of donor polysaccharides focussed on those involved in pectins; Megazyme produced RG-IA and B are from soy bean and potato pectic fibre respectively while AG is from larch wood, either commercially (AG Sigma) or lab made (AG Lab). **Blue** = with beansprout enzyme extract, **red** = enzyme-free control.

4.2 No novel transglycanase activity was detected with non-XGO acceptors

In addition to a range of different potential donor polysaccharides and plant enzyme extracts, I tested transglycanase activity with different radio-labelled oligosaccharides: [^{3}H]Lam$_6$-ol, [^{3}H]Ara$_8$-ol, [^{3}H]Gal$_6$-ol, [^{3}H]Man$_6$-ol, [^{3}H]Xyl$_6$-ol and [^{3}H]Cell$_6$-ol.

No novel homo- or heterotransglycanase activity was detected following 16 h incubation of the tritiated-oligosaccharides with beansprout enzyme extract and various potential donor polysaccharides (Figure 17), with the exception of αA LO : [^{3}H]Ara$_8$-ol where a trace of activity above that of the enzyme-free control was observed (Figure 17c). As these assays were not replicated due to limited tritiated-oligosaccharides, further analysis is required to determine whether the activity observed, indicative of potential novel homotransglycanase activity, is significant. Transglycanse activity with a [^{3}H]XXXGol acceptor was used as a positive control to ensure the reaction had been successful under these conditions.

Some of the acceptor oligosaccharides that I intended to use for this assay were unable to sufficiently bind to paper meaning that removal of unreacted tritiated-oligosaccharide by paper chromatography was not viable. As an alternative, I loaded the transglycanase reaction products onto silica-TLCs, ran them in BAW (2:1:1) and then fluorographed them. This method would potentially allow detection of transglycanase products at the origin due to their high molecular weight (Figure 18).

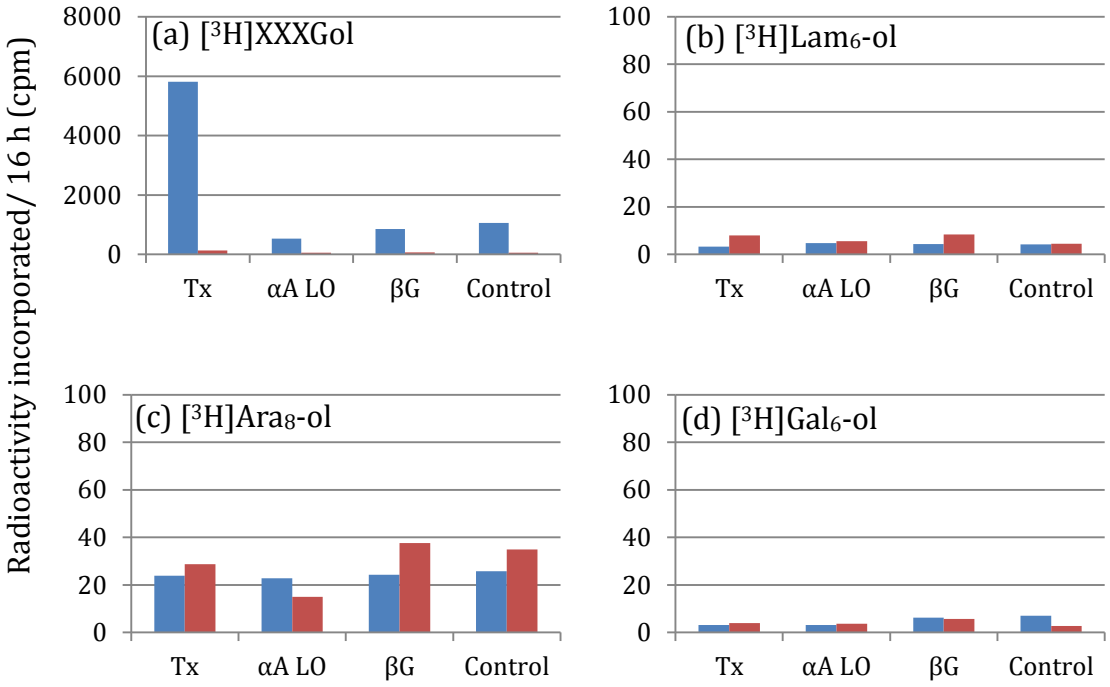


Figure 17: Transglycanase activity observed with a range of tritiated-oligosaccharide acceptors
A range of acceptor oligosaccharides: (a) [^{3}H]XXXGol, (b) [^{3}H]Lam$_6$-ol, (c) [^{3}H]Ara$_8$-ol, and (d) [^{3}H]Gal$_6$-ol were used to study potential transglycanase activities. Reaction mixtures were incubated for 16 h under normal conditions prior to removal of unreacted acceptor via paper chromatography in EAW (10:5:6). Beansprout extract (**blue**) and an enzyme-free control (red) were used.

No transglycase products were observed at the origin of the TLCs for any of the tritiated-acceptors except for the [^{3}H]XXXGol positive control which, as expected, displayed the product of XET activity (Figure 18a). Interestingly, some radioactive-acceptors were degraded by enzymes within the beansprout extract, including [^{3}H]Cell$_6$-ol and [^{3}H]Lam$_6$-ol. Hydrolysis of [^{3}H]XXXGol and [^{3}H]Gal$_6$-ol was also observed but to a lesser extent. A hint of transglycanase activity was detected with a Tx donor polysaccharide and [^{3}H]Gal$_6$-ol in beansprout but this was not observed in any repeated fluorographs and as such is thought to be due to a contaminant (results not shown). Interestingly, the potential αA LO : [^{3}H]Ara$_8$-ol transglycanase activity observed in the radio-assay (Figure 17c) was not detected via fluorography, possibly occurring at undetectable levels for this

assay. Further analysis is required to determine whether this is a true homotransglycanase activity.

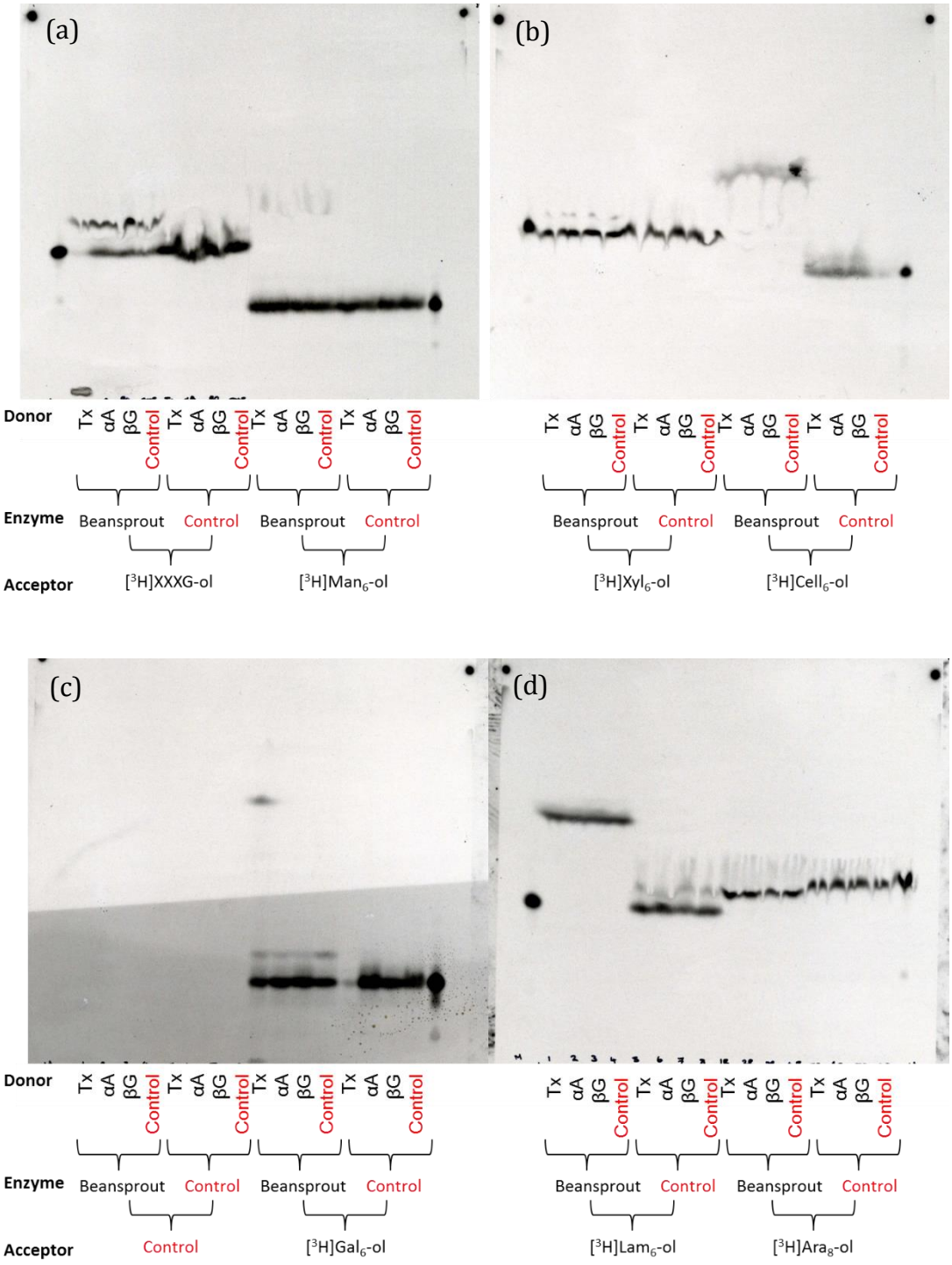


Figure 18: TLCs of transglycanase reactions with a range of different acceptor oligosaccharides

The transglycanase products (10 µg) of different tritiated-acceptors (1 kBq) were fluorographed: (a) [^{3}H]XXXGol and [^{3}H]Man$_6$-ol, (b) [^{3}H]Xyl$_6$-ol and [^{3}H]Cell$_6$-ol, (c) acceptor-free control and [^{3}H]Gal$_6$-ol, (d) [^{3}H]Lam$_6$-ol and [^{3}H]Ara$_8$-ol. Tranglycanase reactions occurred in the presence of either a beansprout enzyme extract or enzyme-free control.

4.3 Determining if apparent novel transglycanase activity is due to XyG contamination

4.3.1 Mild TFA acid hydrolysis to distinguish between furanose- and pyranose-based polysaccharides

Mild acid hydrolysis cleaves furanose but also, to a much lesser extent, pyranose bonds; thus the product of a potential AXE activity (α-arabinan : [^{3}H]XXLGol) would be degraded, as bonds between α-arabinose residues are broken. Hence, no radioactivity would be detected. However, this method does not serve to allow differentiation between tranglycosylation products consisting entirely of pyranose bonds, such as those of XET vs. β-galactan : xyloglucan endotransglycosylase (GXE) reactions. As a result, although contamination of α-arabinan donor polysaccharides with XyG (or other pyranose-based polysaccharide responsible for the observed transglycosylation activity) would be detectable if the transglycosylation product was not lost following mild acid hydrolysis, this would not be possible for β-galactan unless the activity observed was due to contamination with a furanose-based polysaccharide.

As shown in Figure 19, following mild acid TFA hydrolysis, the radioactivity incorporated was retained in all samples compared to the untreated control. This suggests that the activity observed with α-arabinan as the donor was due to contamination with a pyranose-based polysaccharide. Whether this activity, and also the potential GXE activity, was due to XyG contamination (and hence XET activity) was the subject of further analysis.

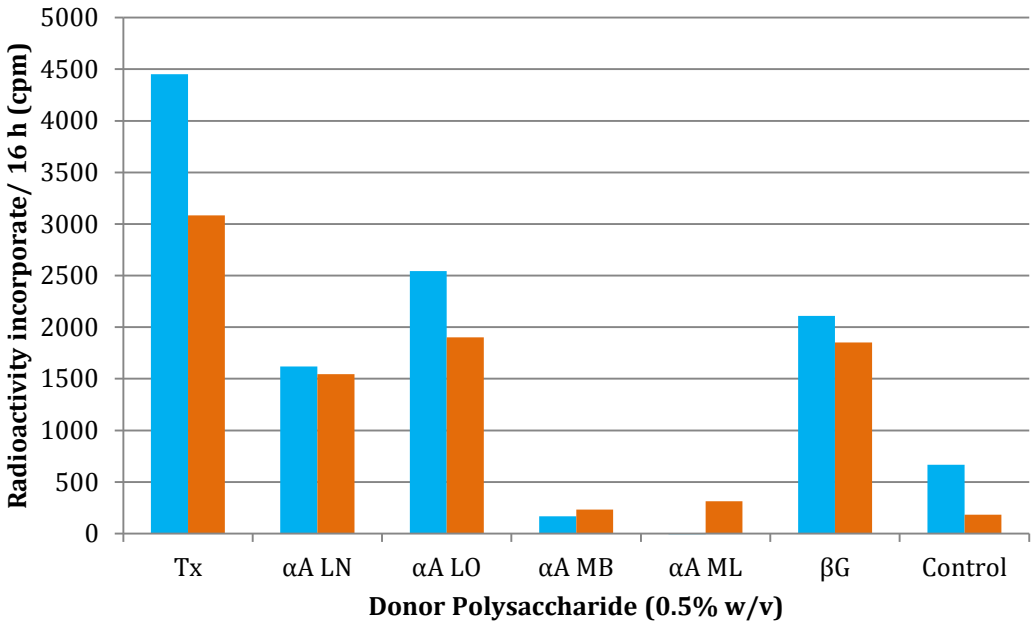


Figure 19: Results of a transglycosylation reaction of beansprout extract following mild-acid hydrolysed potential donor polysaccharides
Mild TFA hydrolysis of donor polysaccharides was carried out for 1 h at 85°C with 0.1 M TFA. Following hydrolysis and removal of TFA, a transglycosylation reaction mixture with beansprout extract and [^{3}H]XXXGol acceptor was incubated for 16 h. Results are cancelled for an enzyme-free control. Untreated donor polysaccharides = **blue**, mild-acid hydrolysed donor polysaccharides = **orange**.

4.3.2 Full-acid hydrolysis to determine monosaccharide content of putative donor polysaccharide samples

All commercial samples of potential donor polysaccharides contained contamination from other polysaccharides (in excess of those stated). Full-acid hydrolysis breaks down most polysaccharides to their constituent monosaccharides. Although this method does not conclusively confirm the contamination of a sample with a specific polysaccharide it can indicate candidates. For example, the presence of xylose and glucose in the correct ratio in an α-arabinan or β-galactan preparation could suggest the presence of XyG.

Figure 20 shows the results of mild-acid and full-acid hydrolysis of a selection of potential donor polysaccharides. Xylose and glucose were present in Tx, αA LN and αA ML samples but were not present at detectable levels in αA LO or αA MB. Whilst αA LN contained xylose and glucose in detectable levels at a ratio which could indicate xyloglucan contamination, and a surprisingly small amount of

arabinose, αA LO showed no detectable xylose or glucose and high levels of arabinose.

Interestingly, αA LN and αA LO showed the highest levels of activity in transglycanase reactions with "α-arabinan" donors yet differ greatly in monosaccharide content. The potential xyloglucan observed in the αA LN sample does not explain why the αA LO sample has almost equal transglycanase activity yet much less (if any) xyloglucan contamination. Interestingly, these samples have similar amounts of galactose, which could indicate GXE activity but this does not explain why no activity is observed with αA MB or αA ML, which contain comparable levels of galactan, despite the latter also potentially containing detectable XyG contamination.

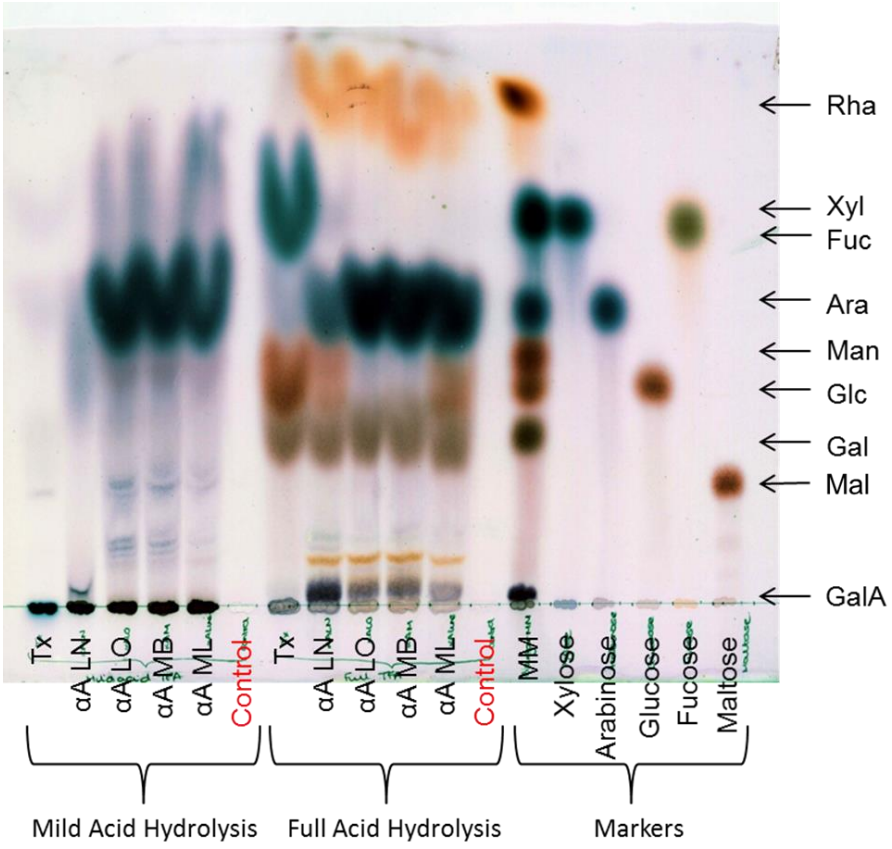


Figure 20: TLC showing products of mild- and full-acid hydrolysis of donor polysaccharides
Potential donor polysaccharides tested include Tx and α-arabinan donor polysaccharides (12.5 µg). Mild-acid and full-acid TFA hydrolysis were as outlined in 2.6.1. Monosaccharide markers (5 µg) were used to allow identification of TFA-released monosaccharides. MM refers to a 'marker mixture' containing GalA, Gal, Glc, Man, Ara, Xyl and Rha. Control refers to succinate (Na^+) buffer (pH 5.5). The TLC was run in EPAW (6:3:1:1) for 3 h.

4.3.3 Driselase digestion of potential donor polysaccharides

Driselase is natural mixture of fungal carbohydrases (including β-xylanase, β-mannanase, laminarinase, cellulase and pectinase) that can be used to release and digest cell wall carbohydrates for analysis. This method could be used to ascertain whether donor polysaccharides are contaminated with XyG as, upon digestion with Driselase, XyG characteristically produces isoprimeverose (IP), a disaccharide of α-D-xylopyranosyl-(1,6)-D-glucose.

Following Driselase digestion of donor polysaccharides (for method see 2.6.3; Figure 21), aliquots of digestion products were loaded onto TLC. As expected, Driselase treatment of Tx yielded high concentrations of isoprimeverose and glucose with traces of xylose. Galactose was also detected at high levels but correlating with that observed following full-acid hydrolysis. With the exception of αA LN, isoprimeverose was not clearly present in any of the α-arabinan or β-galactan donor polysaccharides, although smearing of lanes due to the high salt content of the succinate buffer (buffer A) made this difficult to confirm.

The low level of isoprimeverose detectable following digestion of αA LN is consistent with the xylose and glucose traces observed following full-acid hydrolysis and confirms the contamination of this donor polysaccharide with XyG. However, this does not explain why transglycanase activity with αA LN and αA LO donor polysaccharides are similar. It is possible, even probable, that some if not all of the transglycanase activity observed with αA LN as a donor is due to XET activity rather than AXE but isoprimeverose is not detectable in the digestion products of αA LO. The same is true of βG which shows high transglycanase activity but no detectable XyG contamination. A caveat with TLCs is their limited sensitivity and as such XyG contamination may be present but not detected yet this does not explain how, if the activity observed is due to XET rather than AXE or GXE, it fails to correlate directly with XyG content.

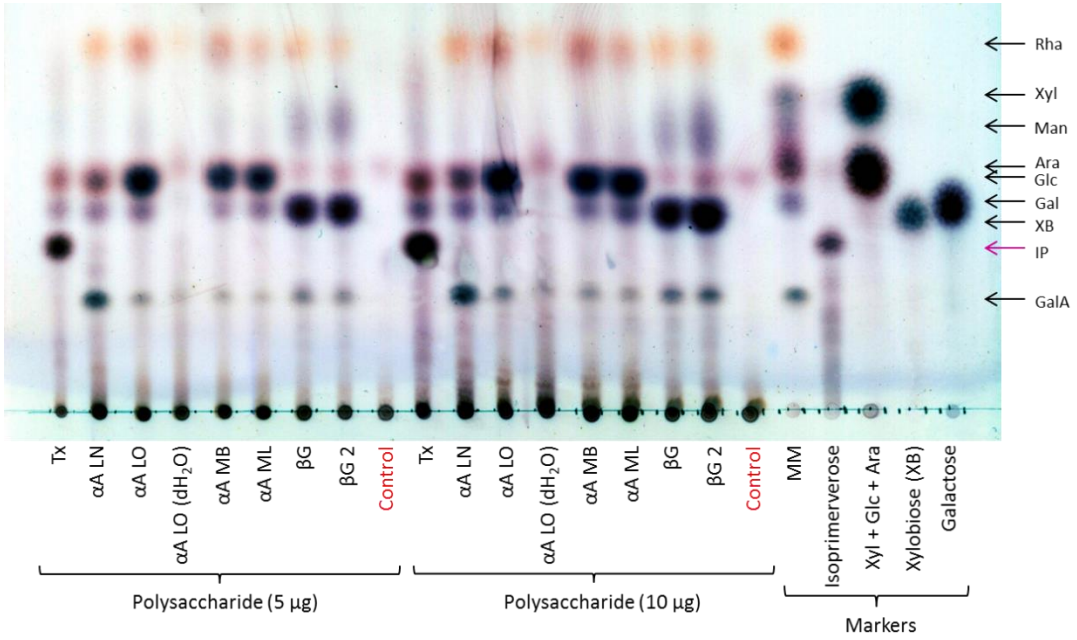


Figure 21: TLC of Driselase digestion products of potential donor polysaccharides
Following loading of two different concentrations of potential donor polysaccharide (5 µg and 10 µg), the TLC was run for 8 h in BAW (4:1:1), thymol stained and developed. 'Control' refers to a polysaccharide-free control. αA LO (dH_2O) the same polysaccharide as in αA LO but resuspended in dH_2O rather than buffer A. Markers (10 µg unless otherwise stated) were used to allow identification of monosaccharides. MM (5 µg) refers to 'marker mixture' which is a combination of Rha, Xyl, Man, Ara, Glc, Gal and GalA. Xylobiose (XB; 1 µg) and isoprimeverose (IP; 1 µg) were also used as markers.

4.3.4 XEG digestion of putative donor polysaccharides

Xyloglucan *endo*glucanase (XEG) cleaves β-1,4-glucose chains producing characteristic XGOs upon digestion of XyG. To determine whether α-arabinan and β-galactan donor polysaccharides were contaminated with exogenous XyG we digested them with a well characterised fungal GH12 XEG (see 2.6.2) and assayed for digestion products qualitatively via TLC. We also used XEG digested potential donor polysaccharides for a radioactive transglycanase assay to determine quantitatively if there was a decrease in radioactive transglycanase product formed, indicating previously observed activity was due XET as a result of XyG contamination.

When XEG digested putative donor polysaccharides were used in the transglycosylation assay, formation of transglycosylation product with all donor polysaccharides decreased to that of the donor-free control (Figure 22).

TLC analysis of XEG digestion products of donor polysaccharides did not show detectable levels of the characteristic XyG digestion products shown on the TLC via XEG digestion of Tx (Figure 23). It is unclear whether, under the conditions used, XEG was capable of digesting α-arabinan or β-galactan to produce a product that was sufficiently large to remain at the origin of the TLC but too small to allow retention of the transglycanase product following paper chromatography.

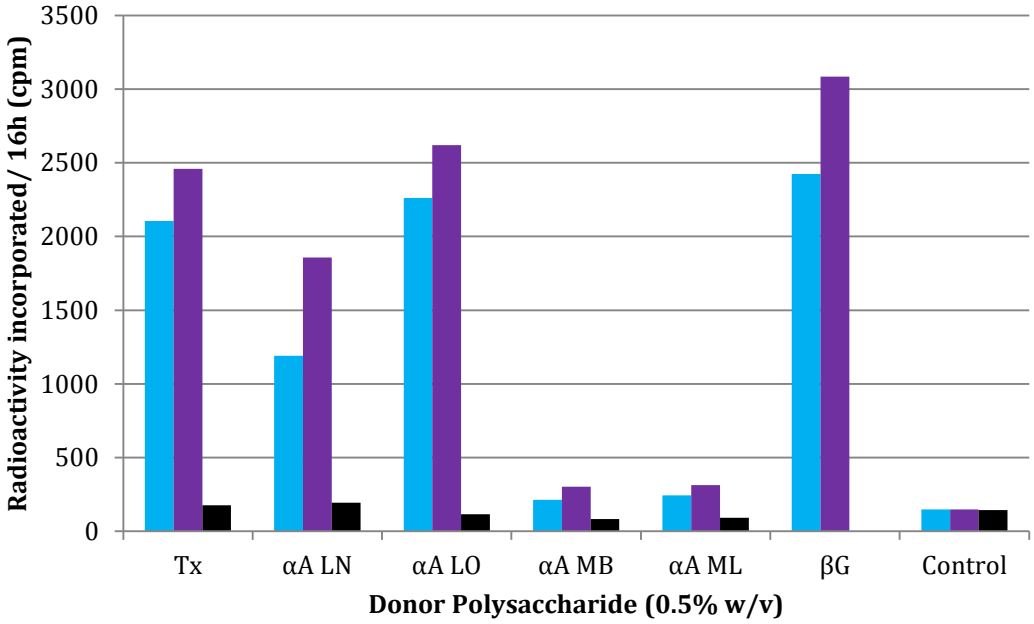


Figure 22: Radioassay of donor polysaccharides before and after XEG digestion

Donor polysaccharides underwent XEG digestion and were then boiled to kill XEG prior to use in a transglycosylation reaction that was incubated for 16 h (**black**). To ensure that boiling of the donor polysaccharide did not affect its ability to act as a donor polysaccharide, a control was boiled but not treated with XEG for comparison (**purple**). The transglycosylation product of the unboiled, undigested donor polysaccharide control was also recorded (**blue**).

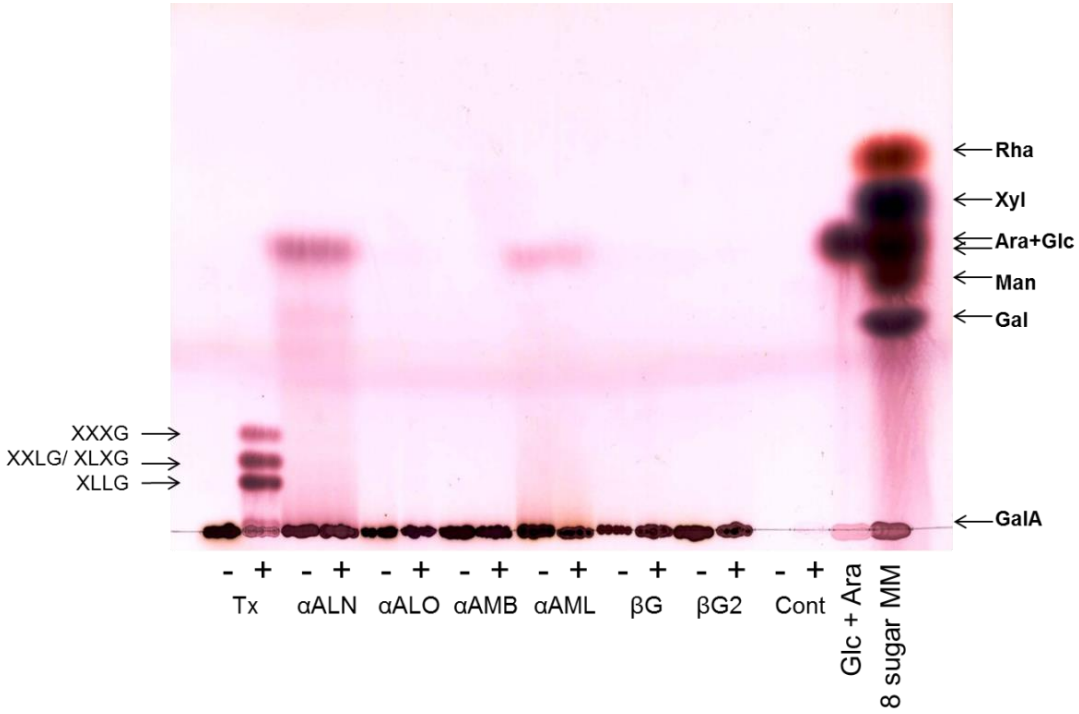


Figure 23: TLC of donor polysaccharides following digestion with XEG
TLC shows undigested (-) and XEG digested (+) potential donor polysaccharides (5 µg). Indicated are the characteristic XEG digestion products of Tamarind XyG, which are not detectable in any other sample. The TLC was run for 8 h in BAW 2:1:1. Markers (10 µg) were used to allow identification of monosaccharides.

4.3.5 Minimum concentration of XyG required for a reaction

Radioactive transglycanase assays have indicated that observed 'novel' transglycanase activity could be due to contamination with XyG and thus represent XET activity. However, degradation products observed on TLCs conflict this as degradation products that could confirm the presence of XyG (such as isoprimeverose following Driselase digestion) have not been detected in all samples conferring potential transglycanase activity. It is possible that the amount of XyG present is too low to be detected by TLC so I aimed to determine the minimum concentration of XyG required for XET activity to be detectable in a transglycanase assay. Also, I aimed to determine the minimum amount of donor polysaccharide that could be detected by TLC. This was done by comparing full-acid hydrolysed potential donor polysaccharides with a dilution series of xylose, glucose and galactose (Figure 24).

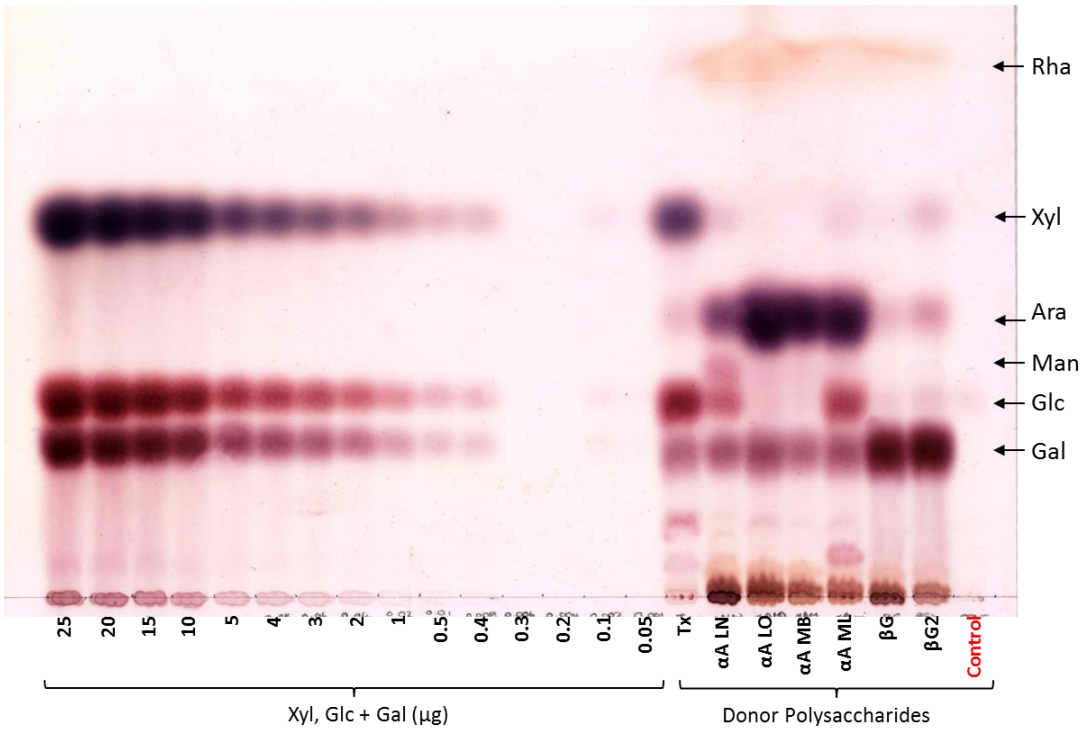


Figure 24: TLC to determine minimum amount of full-acid hydrolysed putative donor polysaccharide detectable by TLC
Potential donor polysaccharides were full-acid hydrolysed and 25 µg of each was loaded onto the TLC which was run in EPyAW (6:3:1:1) and then stained with thymol. 'Control' refers to a polysaccharide-free control. The dilution series, for semi-quantitive analysis of donor polysaccharides, contained equal amounts of Xyl, Glc + Gal.

Figure 24 indicates that donor polysaccharide quantities below 0.4 µg were not detectable by TLC. By comparison between full-acid hydrolysed donor polysaccharides and the dilution series of Xyl, Glc and Gal, semi-quantitative analysis was possible. αA LN had a glucose content of approximately 1-2 µg and a xylose content of approximately 0.5 µg giving a ratio of Glc: Xyl in the region of 2 : 1 and 4 : 1. αA LO and αA MB do not contain xylose or glucose at detectable levels on TLC whilst αA ML has a glucose content of around 4-5 µg but a much lower level of xylose of around 0.4 µg, giving a Glc: Xyl ratio between 10 : 1 and 12.5 : 1. Xyloglucan commonly consists of a structure where ~75% of glucose residues are substituted with xylose thus, if XyG were present as a contaminant in these donor polysaccharides, it does not account for all the glucose detected. Both βG samples had a glucose concentration of around 0.4 µg but differed in

xylose concentration. Xylose was undetectable in βG but was around 0.4 μg in βG2, giving a 1 : 1 ratio of Glc : Xyl.

All potential donor polysaccharides, except the β-galactans, contained a similar concentration of galactose, in the region of 2-3 μg. However, as previously observed, the concentration of xylose, glucose and galactose contamination of donor polysaccharides detected by TLC did not directly correlate with the level of transglycanase activity observed in the radioactive assays.

To determine whether XET activity was detectable with a concentration of XyG below the detectable limits of a TLC, a 5-fold dilution series of Tx was produced, ranging from 5 μg μl^{-1} to 2.56 pg μl^{-1}. All other donor polysaccharides concentrations were kept at 0.5% (w/v; 5 μg μl^{-1}) as with previous assays. Three enzyme extracts were tested: HTG (consensus MXE, see 6.10), 383 (a highly active XET, see 6.2) and beansprout. Transglycanase assays were conducted at 3 different time points – 1 h, 4 h and 16 h – to allow a comparison between XET product produced and that of AXE or GXE to see if they were proportional, an indicator that the activity observed is due to the same enzyme activity (Figure 25).

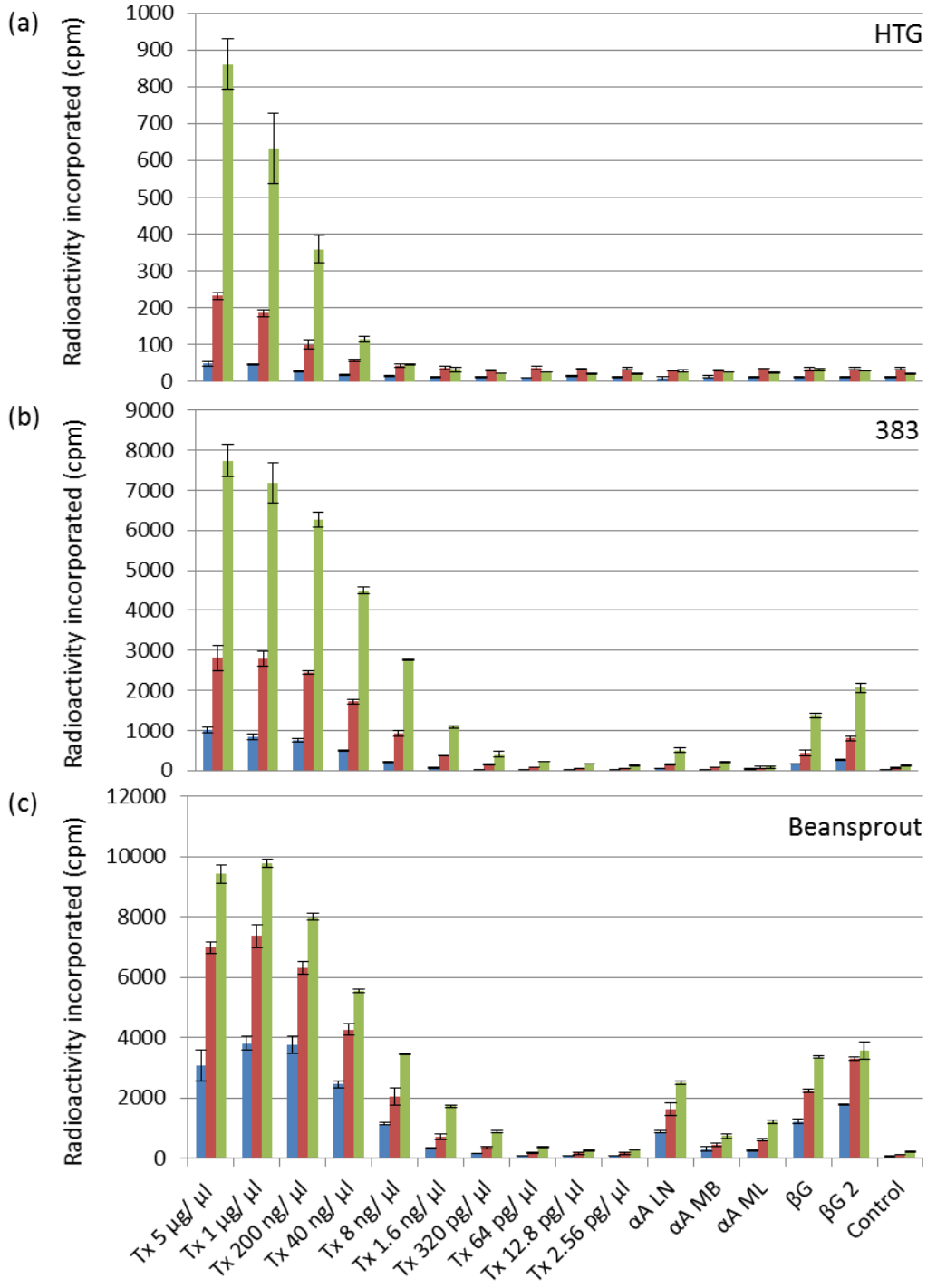


Figure 25: Radio-assay to determine the minimum concentration of XyG required for detectable XET activity

All enzyme extracts (a) HTG, (b) 383 and (c) beansprout were incubated for 1 h (**blue**), 4 h (**red**) or 16 h (**green**) with a [^{3}H]XXXGol (1 kBq) acceptor and various potential donor polysaccharides (5 µg/µl unless otherwise stated). Error bars indicate standard error resulting from triplicate data.

All enzyme preparations showed detectable XET activity but this differed significantly between enzyme extracts. As expected, HTG – a predominant MXE – produced the lowest amount of XET product with a maximum incorporation of 854 cpm following 16 h incubation with 5 μg μl^{-1} Tx (Figure 25a). Beansprout was the most active extract for XET activity, reaching full incorporation of radioactivity during 16 h incubation (Figure 25b). It was over twice as active as 383 (Figure 25c) when 1 h and 4 h incubation periods were compared. HTG did not show any significant transglycanase activity with any donor polysaccharides other than Tx, even after 16 h incubation. Meanwhile, 383 and beansprout both exhibited transglycanase activity with αA LN and both βG donors. Beansprout also showed notable activity with αA MB and αA ML.

When 383 was incubated with αA LN, radioactivity incorporated was significantly higher than that of the control. For the same donor polysaccharide and incubation times, beansprout showed much higher radioactivity incorporated than 383. These results correlate with a contamination of αA LN with XyG in the region of 344 pg μl^{-1} to 6.47 ng μl^{-1} (Table 3).

Incubation of 383 or beansprout extracts with βG for 1 h, 4 h and 16 h indicated significant transglycanase activity which correlated with a possible XyG contamination in the range of 2.76 ng μl^{-1} to 11.5 ng μl^{-1}. The activity observed with βG2 as the donor polysaccharide for 383 and beansprout correlated with a potential XyG content between 8.24 ng μl^{-1} and 23.2 ng μl^{-1}. Results for αA MB and αA ML indicate XyG contamination in the range of 75.1 pg μl^{-1} to 985 pg μl^{-1} and 12.2 pg μl^{-1} to 881 pg μl^{-1} respectively (Table 3).

Transglycanase activity in the beansprout donor-free control was over twice that of the donor-free control for 383 (Figure 25). Although this could indicate a higher amount of endogenous XyG in the beansprout extract, both extracts have been shown to produce transglycosylation products which would result from a similar amount of XyG contamination due to the differences in the enzymatic activity of these enzyme extracts. This, along with the fact that beansprout has more transglycanase activity at lower known Tx concentrations, suggests it

contains more active XET, either due to a greater concentration of XET-active XTHs present in solution or the XTHs present being more XET-active, rather than a higher concentration of contaminating XyG.

Table 3: Summary of potential XyG contamination detected for each polysaccharide

By comparison of transglycanase activity detected for each potential donor polysaccharide with that detected for varying amounts of Tx it was possible to calculate an approximate amount of XyG contamination that would correlate with the activity observed. Potential XyG contamination was calculated for (a) 383 and (b) BS and would include any endogenous XyG within the enzyme extract. Use of a donor-free control allowed endogenous XyG and contaminating XyG to be distinguished.

(a) 383

Donor	1 h	4 h	16 h
αA LN	696 pg μl^{-1}	344 pg μl^{-1}	492 pg μl^{-1}
αA MB	168 pg μl^{-1}	84.1 pg μl^{-1}	75.1 pg μl^{-1}
αA ML	383 pg μl^{-1}	84.1 pg μl^{-1}	12.2 pg μl^{-1}
βG	5.18 ng μl^{-1}	2.76 ng μl^{-1}	3.63 ng μl^{-1}
βG2	13.3 ng μl^{-1}	8.50 ng μl^{-1}	8.24 ng μl^{-1}
Control	57.3 pg μl^{-1}	50.7 pg μl^{-1}	28.2 pg μl^{-1}

(b) Beansprout

Donor	1 h	4 h	16 h
αA LN	6.47 ng μl^{-1}	5.88 ng μl^{-1}	4.91 ng μl^{-1}
αA MB	985 pg μl^{-1}	457 pg μl^{-1}	270 pg μl^{-1}
αA ML	733 pg μl^{-1}	826 pg μl^{-1}	881 pg μl^{-1}
βG	11.5 ng μl^{-1}	10.8 ng μl^{-1}	9.69 ng μl^{-1}
βG2	22.4 ng μl^{-1}	23.2 ng μl^{-1}	11.2 ng μl^{-1}
Control	58.5 pg μl^{-1}	32.6 pg μl^{-1}	12.9 pg μl^{-1}

By investigating the minimum concentration of XyG required for XET activity to be detectable in our radio-assay I have shown that concentrations which result in activity were below the levels detectable by TLC. As a result, although XyG degradation products were not detected on TLC previously this does not prove that the donor polysaccharides are not contaminated with XyG. Each donor polysaccharide was shown to produce transglyanase activity which could correlate with a trace contamination of XyG. Interestingly, despite xylose and glucose being detectable in some samples on the TLC, the level of XyG that would correlate with the observed transglycanase activity is at undetectable limits suggesting that the majority of xylose and glucose observed in these samples is from a different source. Although this strongly indicates that XyG could act as the contaminant despite it being present at such low concentration, it does not conclusively prove that the contaminant is XyG.

4.3.6 Galactanase digestion

To determine the presence of GXE activity, transglycanase reaction products were galactanase digested (see 2.6.4). A range of different incubation times and galactanase concentrations were tested to find conditions under which β-galactan was digested but Tx was not (Figure 26). Optimal conditions were 100 min with an enzyme concentration of 1% (4.40 U mg^{-1}). Although higher concentrations of enzyme were equally effective, they were decided against due to their high ammonium sulphate concentration.

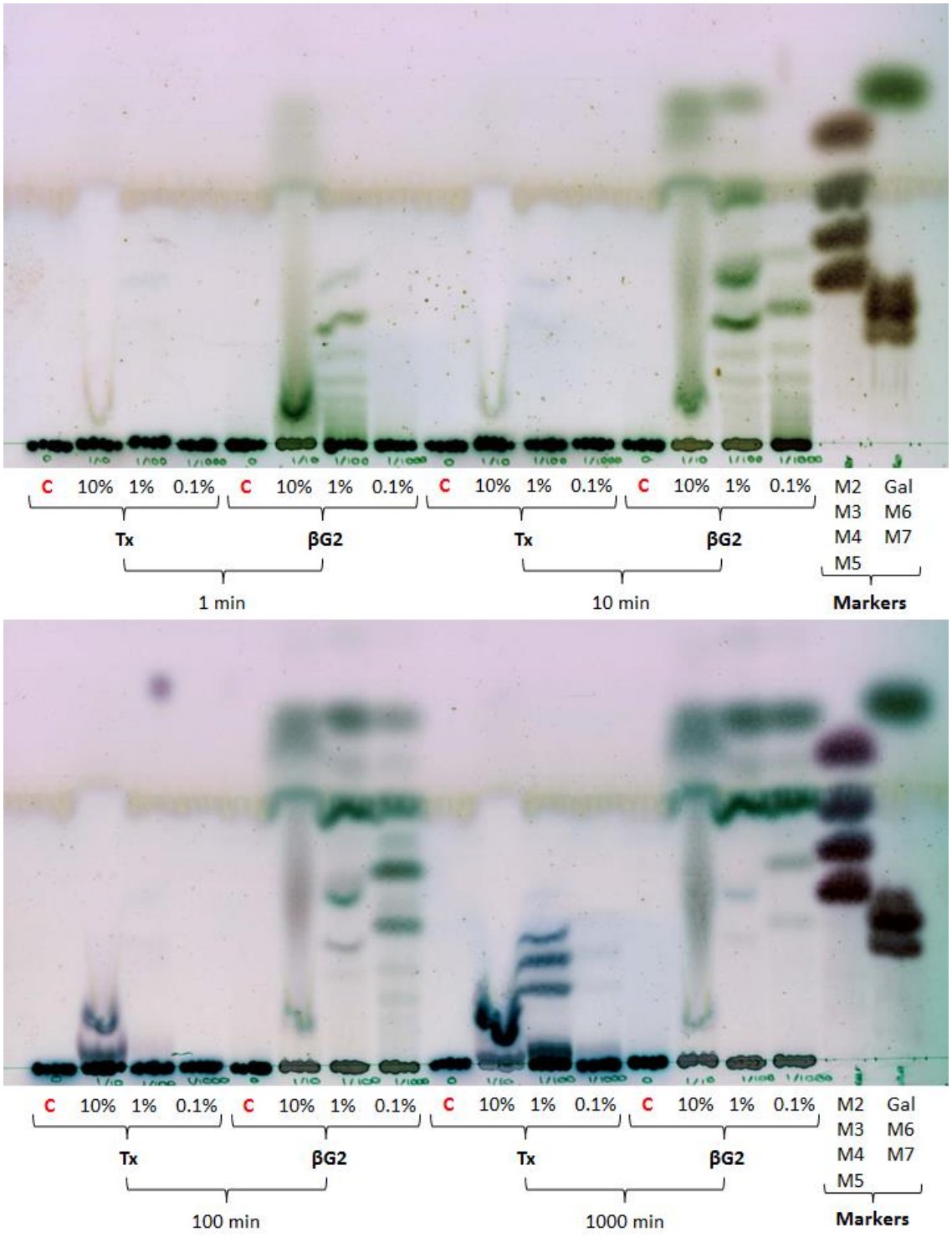


Figure 26: TLCs of galactanase digestion products of Tx and βG produced under varying conditions

Galactanase digested polysaccharides (10 µg) and markers (5 µg) – maltose ladder oligosaccharides and galactose – were loaded onto TLCs that were then run in EPyAW (6:3:1:1) for 4 h prior to thymol staining. Polysaccharides were digested with 10%, 1% and 0.1% (v/v) concentrations of galactanase for 1 min, 10 min, 100 min and 1000 min. 'C' corresponds to a donor polysaccharide sample not digested by galactanase.

The radioactive transglycosylation reaction was incubated for 16 h at room temperature prior to digestion with a 1% solution of galactanase (4.40 U ml^{-1}) for 100 min. Results of the radioactive assay (Figure 27) showed a dramatic decrease in transglycanase activity but it remained significantly higher than that of the donor-free control.

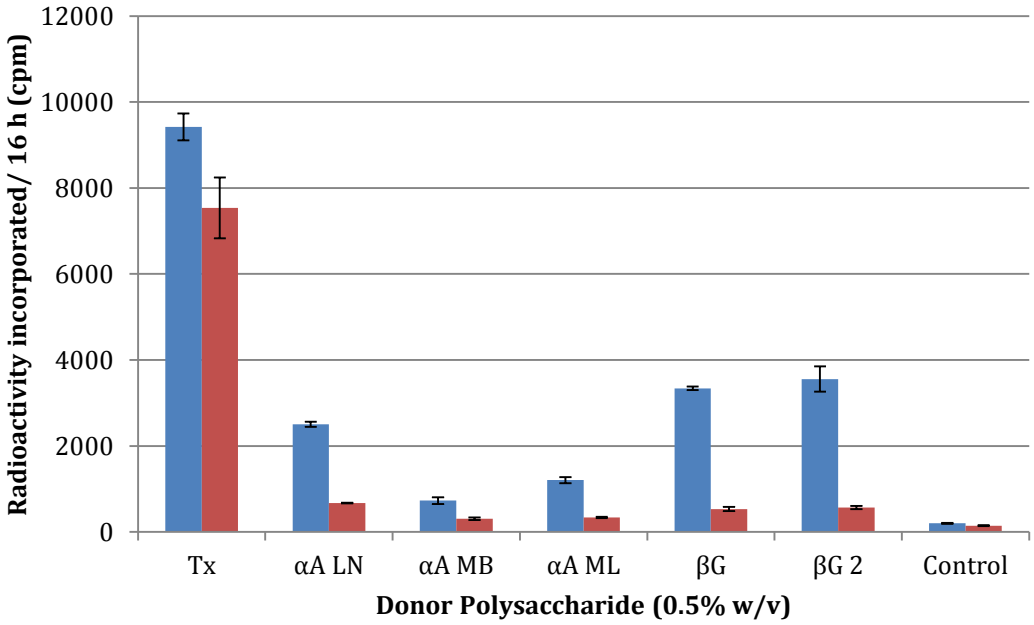


Figure 27: Transglycanase product detected with and without galactanase digestion
Radioactive transglycanase product detected for undigested (**blue**) and galactanase digested (**red**) reaction mixtures. Samples were assayed for transglycanase activity following incubation with a [^{3}H]XXXGol acceptor (1 kBq), potential donor polysaccharide and beansprout enzyme extract. Error bars indicate standard error from triplicate results.

According to these results, the transglycanase activity detected could have, in part, been due to the presence of a β-galactan donor polysaccharide but, as some activity remained, it could not have been due to this proposed GXE activity alone. I hypothesised that the most probable explanation for this incomplete loss of transglycanase activity was that the activity observed was due to XET but, as only a trace of XyG would have been present as a contaminant of the donor polysaccharide, some of it could have been digested by galactanase but at a level that would not have been detectable by TLC.

To investigate this hypothesis I repeated the transglycanase reactions. However, once the reaction had been stopped and the formic acid removed, I supplemented the mixture with Tx (20 µg), which was comparable to the amount digested for the TLC analysis. Under these conditions, if the galactanase digested some XyG rather than β-galactan, it is less likely to digest the trace amount of potentially radio-labelled XyG. As a result, if activity persists then the activity is due to XET and not GXE (Figure 28)

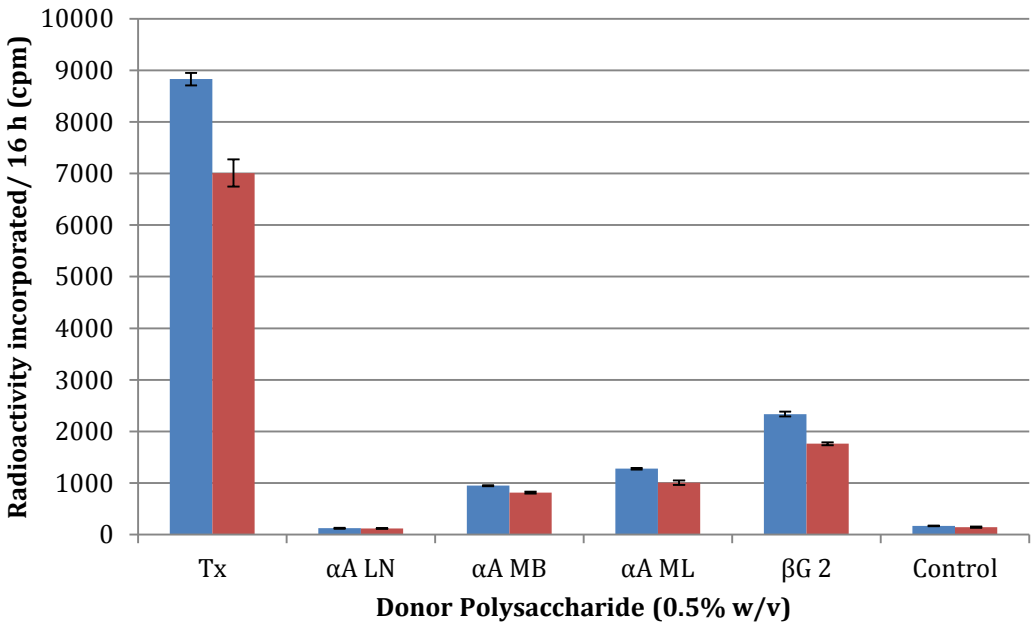


Figure 28: Transglycanase product retained following supplementation with excess Tx and galactanase digestion
Radioactive transglycanase product detected for undigested (**blue**) and galactanase digested (**red**) reaction mixtures. Error bars indicate standard error from triplicate results.

Addition of excess Tx following completion of the transglycosylation reaction and prior to galactanase digestion resulted in a higher retention of transglycosylase product (Figure 28). This suggested that the supplementary added Tx protected much of the trace radio-labelled XET product from digestion by galactanase. However, further analysis was required to determine whether the slight decrease in activity still observed was due to digestion of a GXE product.

4.3.7 Cellulase digestion

To determine whether GXE activity occurred in addition XET activity following transglycosylation assays with β-galactan polysaccharides I aimed to digest XyG with a XyG-digesting cellulase and determine if any transglycanase product was retained. To determine the optimum conditions which would allow digestion of XyG but not β-galactan a range of different incubation times and cellulase concentrations were tested (Figure 29). Optimal conditions were 100 min with an enzyme concentration of 1% (v/v).

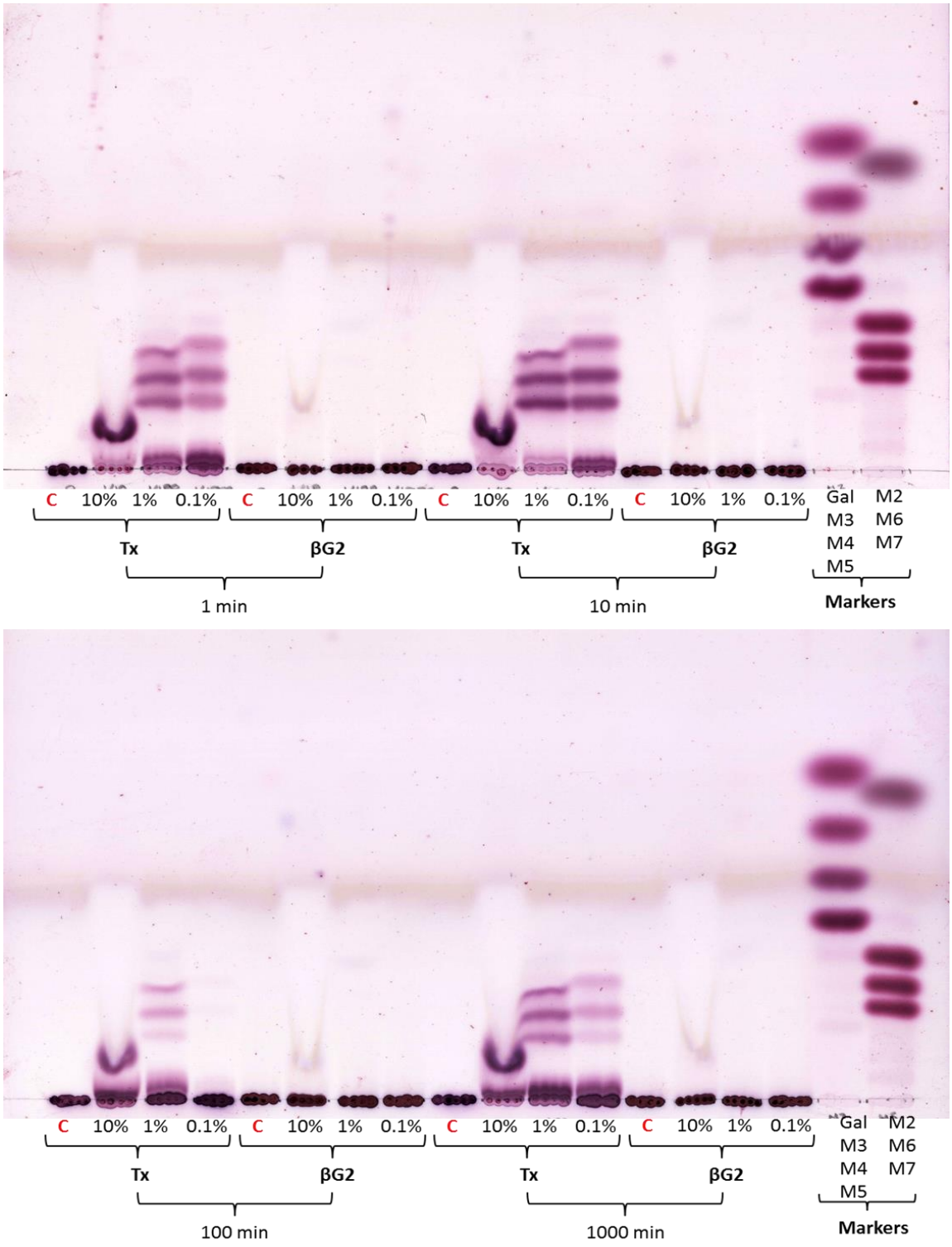


Figure 29: TLCs of cellulase digestion products of Tx and βG produced under varying conditions

Cellulase digested polysaccharides (10 µg) and markers (5 µg) – a maltose ladder (M2-M7) and galactose – were loaded onto TLCs which were run in EPyAW (6:3:1:1) for 4 h prior to thymol staining. Polysaccharides were digested with 10%, 1% and 0.1% (v/v) concentrations of cellulase for 1 min, 10 min, 100 min and 1000 min. The M7 marker mixture has been found to be contaminated, most likely with M8.

I again conducted a radioactive transglycosylation reaction but this time supplemented the potential donor polysaccharides with non-radioactive β-galactan in an attempt to protect any trace GXE products from cellulase digestion, even though, as before, this activity was not detectable by TLC under these conditions. The potential donor polysaccharide was digested with cellulose prior to the transglycanase reaction as to ensure that a loss of activity was not due to digestion of the [^{3}H]XXXGol acceptor. Cellulase digestion of transglycanase products caused a complete loss of transglycosylation product, with only transglycanase products formed with Tx significantly above that of the control (Figure 30), most likely because it had the most XyG to be degraded and under these conditions digestion may not have been as complete as indicated by TLC.

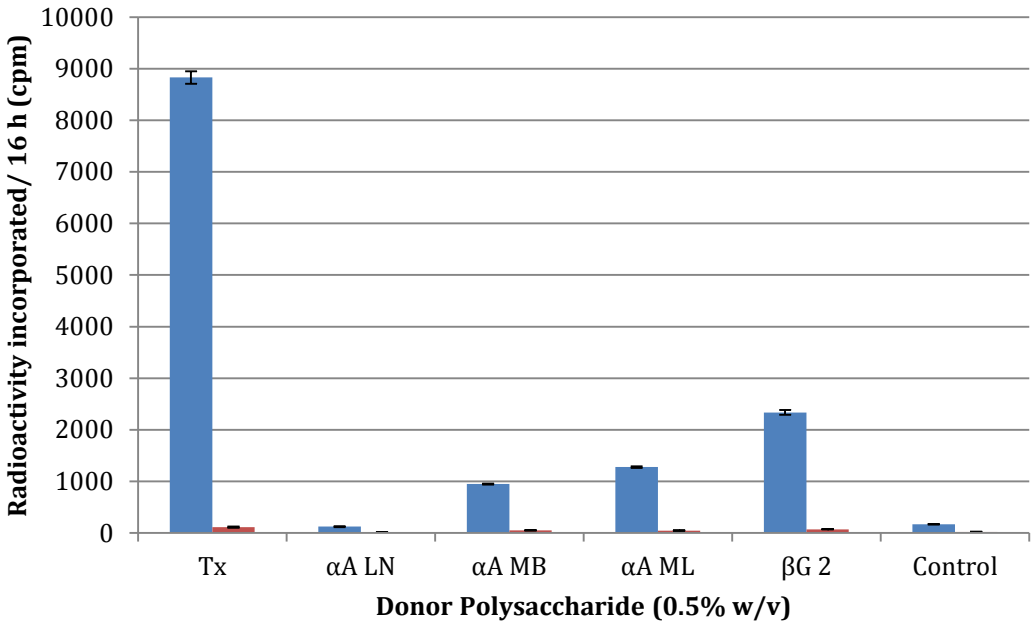


Figure 30: Transglycanase product formed by a radio-assay with donor polysaccharides following cellulase digestion in the presence of non-radioactive galactan
Undigested (**blue**) and digested (**red**) potential donor polysaccharides were incubated with a [^{3}H]XXXGol acceptor and beansprout enzyme extract for 16 h. Error bars indicate standard error calculated for triplicate results.

In conclusion, the apparent novel transglycosylation activity observed with α-arabinan and β-galactan donors has been shown to be due to contamination with trace amounts of XyG. It also strongly indicates that there is no transglycosylation

activity occurring with a [^{3}H]XXXGol acceptor and α-arabinan or β-galactan donor, even in the case of RG-I and arabinogalactan.

5 Results: Broad-spectrum transglycanase assays incorporating an increased range of enzyme extracts and donor polysaccharides

5.1.1 XET assay to test plant extracts for enzyme activity

The presence of known transglycanase activities have been shown to differ between different plant species. For example, appreciable MXE activity has only been conclusively proven in *Equisetum* whereas XET activity is ubiquitous throughout land plants. A wide range of plant samples which act as representative samples of the majority of the Embryophyta were used to create enzyme extracts which were subsequently assayed for XET activity with a xyloglucan donor (Tx) and [^{3}H]XXXGol radio-labelled acceptor. All enzyme extracts displayed significant XET activity, indicating that samples were enzymatically active, but the extent of which varied dramatically between samples despite a common extraction ratio of 2:1 (buffer A (ml)/ dry weight (g)) (Figure 31).

Beansprout, asparagus, crocus, snowdrops and *E. arvense* displayed the highest levels of XET activity. Cauliflower, Arabidopsis, chicory, tomato (skin and outer pericarp) and *H. lanatus* also showed high XET activity whereas activity detected in *E. fluviatile* and *Marchantia polymorpha* were much lower.

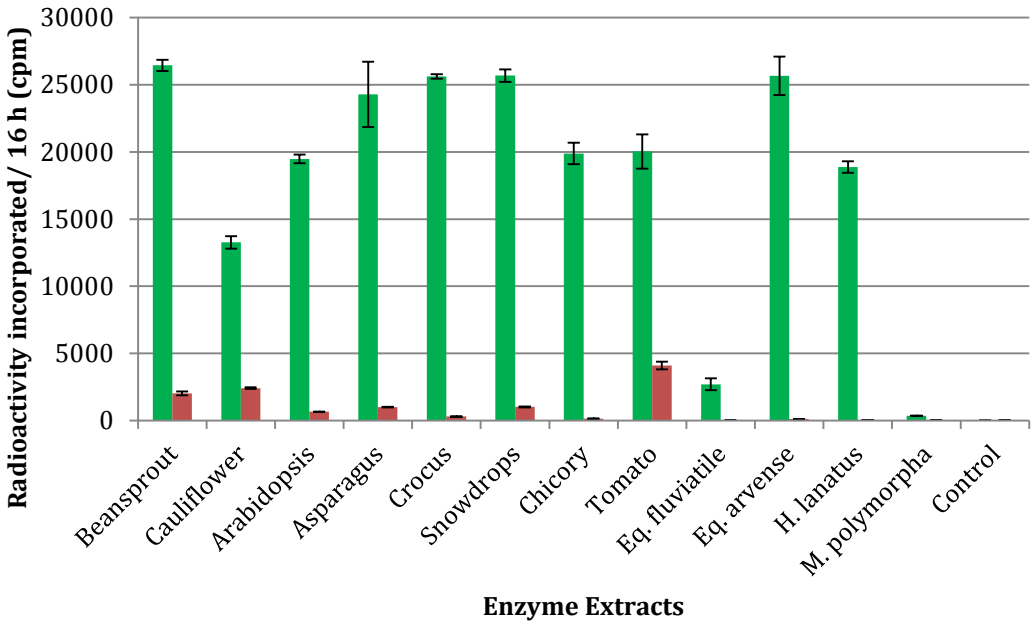


Figure 31: XET activity of enzyme extracts of plant species
Enzyme extracts were incubated, in triplicate, for 16 h at room temperature with 1 kBq [^{3}H]XXXGol acceptor and tamarind XyG donor (0.5% w/v) before being assayed for production of ^{3}H-labelled XET product. All enzyme extracts displayed significant XET activity (**green**) compared to that of the donor-free control (**red**). Error bars indicate standard error of triplicate results.

5.1.2 Full-acid hydrolysis of potential donor polysaccharides

Full-acid hydrolysis was conducted on all potential donor polysaccharides to allow observation of the monosaccharide composition and aid identification of potential contaminants (Figure 32). Xylose was present in Tx, arabinoxylan, glucuronoxylan and xylan as expected but also in small amounts as contaminants in MLG, βG2 and β-(1,3)-glucan.

Arabinose was detectable in trace amounts in Tx, βG2 and galactomannan while αA MB, and β-mannan contained galactose contaminants. The galatose observed in Tx, RG-IB and pectin, although not part of the main chain of the polysaccharide is likely to be due to the presence of galactose-containing side chains.

HEC was shown to contain glucose and hydroxyethylglucose (which runs slightly faster than mannose) and potential traces of arabinose but smearing of this made it difficult to detect other possible contaminants. βG2, αA MB and RG-IB all contained GlcA and GalA while pectin contained only GalA. The amount of GalA

detected in pectin was surprisingly small. Both glucuronoxylan and xylan had other bands above GlcA and GalA which could correspond to 4-*O*-methylglucuronic acid.

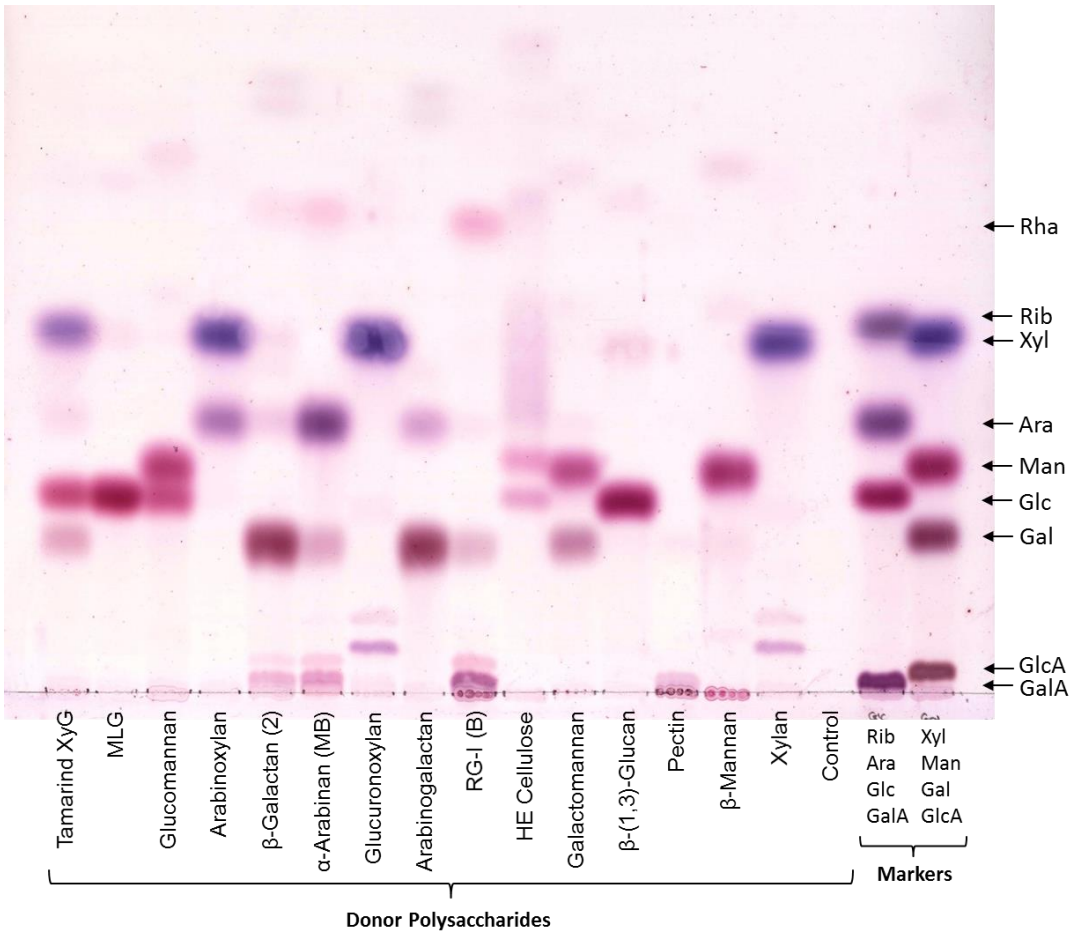


Figure 32: Full-acid hydrolysis of all potential donor polysaccharides
Polysaccharides were hydrolysed by 2 M TFA for 1 h at 120°C. TFA was removed and the polysaccharide resuspended in dH_2O to give a final concentration of 0.5% (w/v). Of this, 2 µl (10 µg) of polysaccharide was loaded onto a silica-TLC and run for 4 h in EPyAW (6:3:1:1). The dried TLC was stained with thymol and developed.

Although the full-acid hydrolysis of donor polysaccharides does not indicate any potential contamination which could result in known transglycanase activity it has been shown previously (4.3.5) that TLCs are not sufficiently sensitive to detect trace amounts of contaminants, such as XyG, which can still support detectable transglycanase activity. Despite this, full-acid hydrolysis of donor

polysaccharides in this way is still useful in allowing an insight into their monosaccharide content and will act as a reference point for future work if potential novel transglycanase activity is not a result of XyG contamination.

5.1.3 Broad-spectrum transglycanase radio-assay with a range of donor and enzyme extract combinations

As in 3.2.2, the method in 2.7.4 was modified to include a greater range of donor polysaccharides: hydroxyethyl cellulose (HEC) as a representative cellulosic polysaccharide, hemicellulosic polysaccharides including tamarind xyloglucan (Tx), mixed-linkage glucan (MLG), β-(1,3)-glucan, glucomannan, arabinoxylan, glucuronoxylan, β-mannan and xylan, and pectic polysaccharides such as β-(1,4) galactan (βG2), α-(1,5)-arabinan (αA MB), arabinogalactan (AG Sigma), RG-I (RG-IB) and pectin (homogalacturonan). An enzyme-free control allowed distinction between significant, potentially novel transglycanase activity with the specific donor polysaccharide and that resulting from the presence of endogenous polysaccharides within the enzyme extract (Figure 33 and Table 4).

5.1.3.1 Significant XET activity is lost following storage of enzyme extracts

As shown in 5.1.1, all enzyme extracts produced detectable XET activity. However, following storage at -20°C, all enzyme extracts lost a significant proportion of their XET activity (Figure 33 and **Table 4**): a decrease in over 60% of the original (except *M. polymorpha* which lost 44.3% activity but had undergone fewer freeze-thaw cycles than the rest). XET activity loss varied between enzyme extracts and, most notably, chicory XET activity decreased from 25 617 cpm to 466 cpm, retaining only 2.3% of its original XET activity for the same incubation period. Other enzyme extracts that showed a particularly dramatic decrease in XET activity following freezing were *H. lanatus*, *E. arvense*, *E. fluviatile*, cauliflower and crocus. More stable XET activity was observed in Arabidopsis, snowdrop, tomato (skin and outer pericarp), beansprout and asparagus. Despite this loss of XET activity, it was still possible to detect XET and

hence presumably also any potential novel transglycanase activities in all enzyme extracts using this radio-assay.

5.1.3.2 Pectic polysaccharides

All samples yielded significant production of transglycanase product with β-galactan as the donor polysaccharide Figure 33. As explained in depth in section 4, transglycanase activity observed with β-galactan (or indeed α-arabinan) are due to contamination of the donor polysaccharide with trace amounts of XyG and, therefore, are not indicative of novel transglycanase activity. Possible pectin : [^{3}H]XXXGol activity was detectable in all samples except *M. polymorpha*; highest in Arabidopsis, beansprout and snowdrop. Interestingly, possible activity with the other tested pectic polysaccharides was observed at low levels with arabinogalactan in beansprout, cauliflower and tomato and with RG-I for tomato only.

5.1.3.3 Cellulosic polysaccharide

As explained in 3.2.2, activity observed with HEC could be due to XET activity due to similarities in HEC and XyG structure. It has been shown that XET activity can be reliant on specific xylose residues bound to the β-(1,4)-linked glucose backbone of xyloglucan for recognition within the enzyme's acceptor binding site (1.4.1). If this is an absolute requirement for all XETs then HEC-oligosaccharides would be unable to act as acceptors for XET but it is conceivable that they would be able to act as donor polysaccharides due to the more relaxed requirements of the donor binding site. Activity with HEC as the donor polysaccharide was high in all enzyme extracts but varied relative to the overall XET activity observed potentially indicating a novel transglucanase activity (Figure 33 and Table 4).

5.1.3.4 Hemicellulosic polysaccharides

5.1.3.4.1 Glucans

As expected, high MXE activity was detected in both *E. arvense* and *E. fluviatile* extracts, actually in excess of the XET activity observed (Figure 33 and Table 4). Notable MXE activity was observed in beansprout, asparagus, crocus, snowdrop, *H. lanatus* and tomato enzyme extracts whilst Arabidopsis, cauliflower and *M. polymorpha* also had MXE activity that was low but significantly above that of the control. When β-(1,3)-glucan was used as the donor polysaccharide, transglycanase activity was highest in cauliflower but also significantly above that of the enzyme-free control in beansprout and tomato samples.

5.1.3.4.2 Xylans

Potential xylan : [^{3}H]XXXGol activity was detectable above background activity in asparagus, beansprout, *E. arvense*, snowdrop and tomato whilst transglycanase activity with an arabinoxylan donor was frequently low but significantly above background in Arabidopsis, asparagus, beansprout, cauliflower, chicory, *E. arvense*, *E. fluviatile*, snowdrop and tomato. No activity was detectable in crocus, *H. lanatus* or *M. polymorpha* samples. Activity with glucuronoxylan meanwhile was observed at the highest levels in *E. fluviatile* but was higher than that of the control in *E. arvense*, *H. lanatus* and snowdrop (Figure 33 and Table 4).

5.1.3.4.3 Mannans

Potential glucomannan : [^{3}H]XXXGol (donor : acceptor) activity was relatively high in Arabidopsis, asparagus, beansprout, chicory, *E. arvense*, *H. lanatus*, snowdrop and tomato samples but was detectable at levels above that of the control in cauliflower, *E. fluviatile* and *M. polymorpha*. Other mannan-based donor polysaccharides – galactomannan and β-(1,3)-mannan – both gave detectable activity in tomato. Whilst galactomannan gave significant activity in Arabidopsis, beansprout and *H. lanatus*, and above background activity for asparagus, chicory, crocus, *E. arvese* and snowdrop, activity with β-mannan was

much lower. When β-mannan was used as the donor polysaccharide, transglycanase activity was low but significantly above that of the enzyme-free control for asparagus, beansprout, cauliflower, and *E. arvense* (Figure 33 and Table 4).

Although transglycanase activity in the presence of a donor-free control varied between samples due to endogenous polysaccharides – it was particularly high for asparagus (785 cpm), beansprout (855 cpm) and cauliflower (845 cpm) – the enzyme-free control consistently produced transglycanase activity for all donor polysaccharides, equal to that of background (Figure 33 and Table 4).

The broad-spectrum radio-assay of a range of different donor polysaccharide and enzyme extract combinations yielded a lot of positive results which could indicate potential novel transglycanase activity. However, it is important to be critical about obtaining so many positive results as, due to time constraints, it has been impossible to distinguish between real activity and that resulting from contamination of the putative donor polysaccharides. Despite this, the prevalence of an observed activity can indicate the results more likely to be a true representation of novel transglycanase activity, thus highlighting preferential donor polysaccharides for further analysis. Although the donor polysaccharides conferring activity in all enzyme extracts may be due to novel transglycanase activity (e.g. HEC), those that produce activity in a limited number of samples (e.g. AG in beansprout, cauliflower and tomato) are less likely to be due to contamination with XyG as all enzyme extracts are capable of XET activity (although they may differ in their ability to produce XET activity with trace amounts of XyG). Further analysis is required to determine which, if any, donor polysaccharides support novel transglycanase activities but this broad-spectrum assay has acted as a suitable indicator of potential candidates.

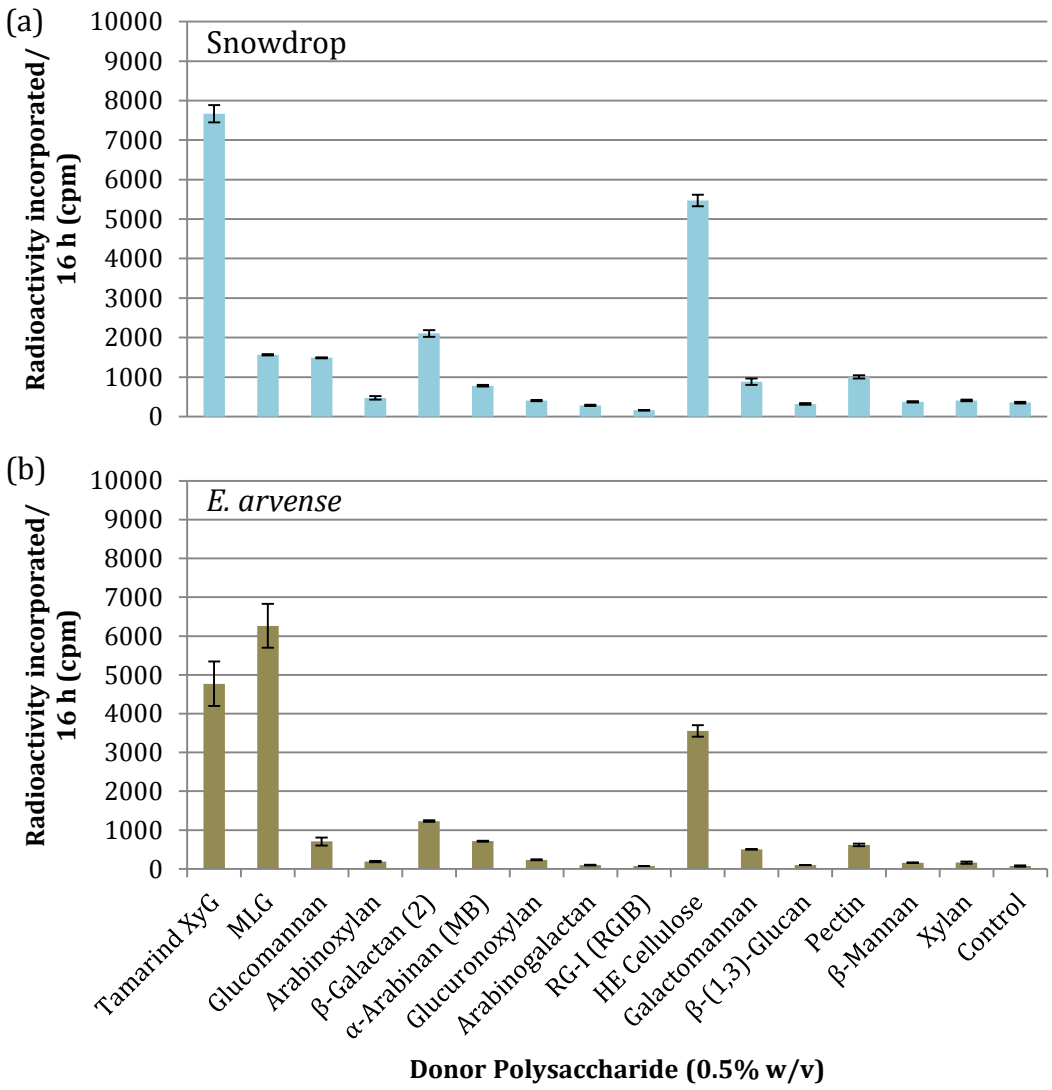
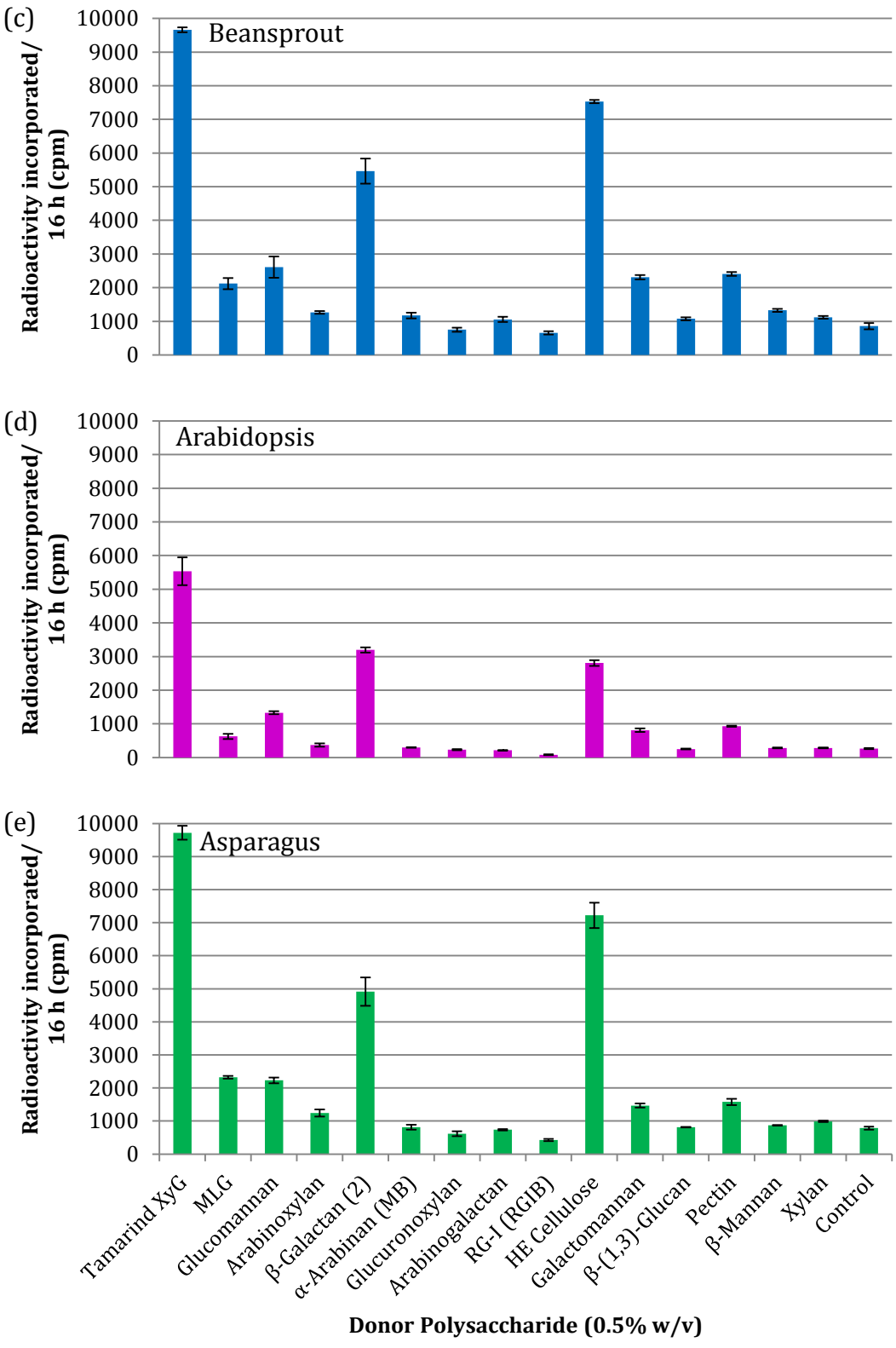
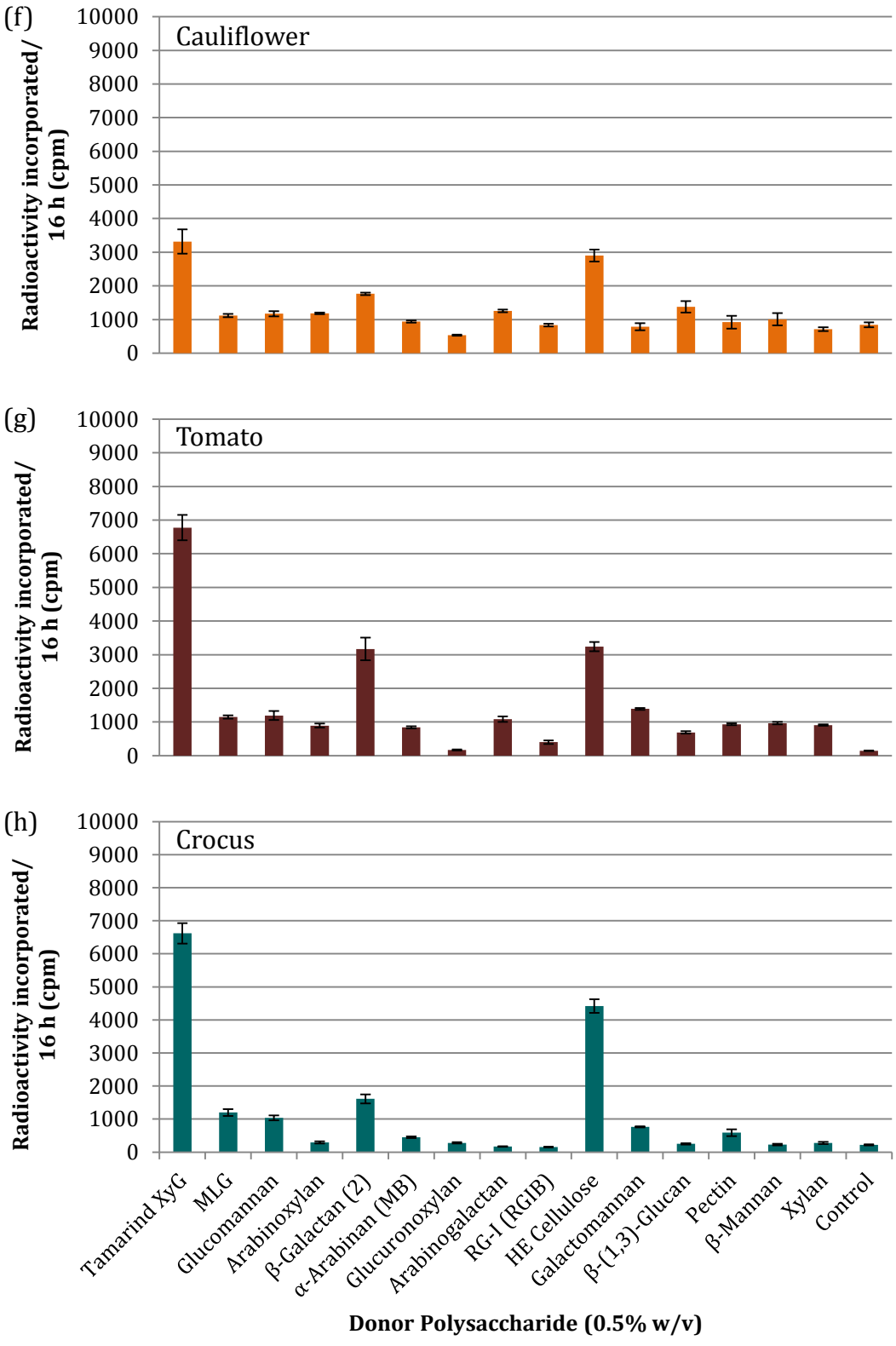
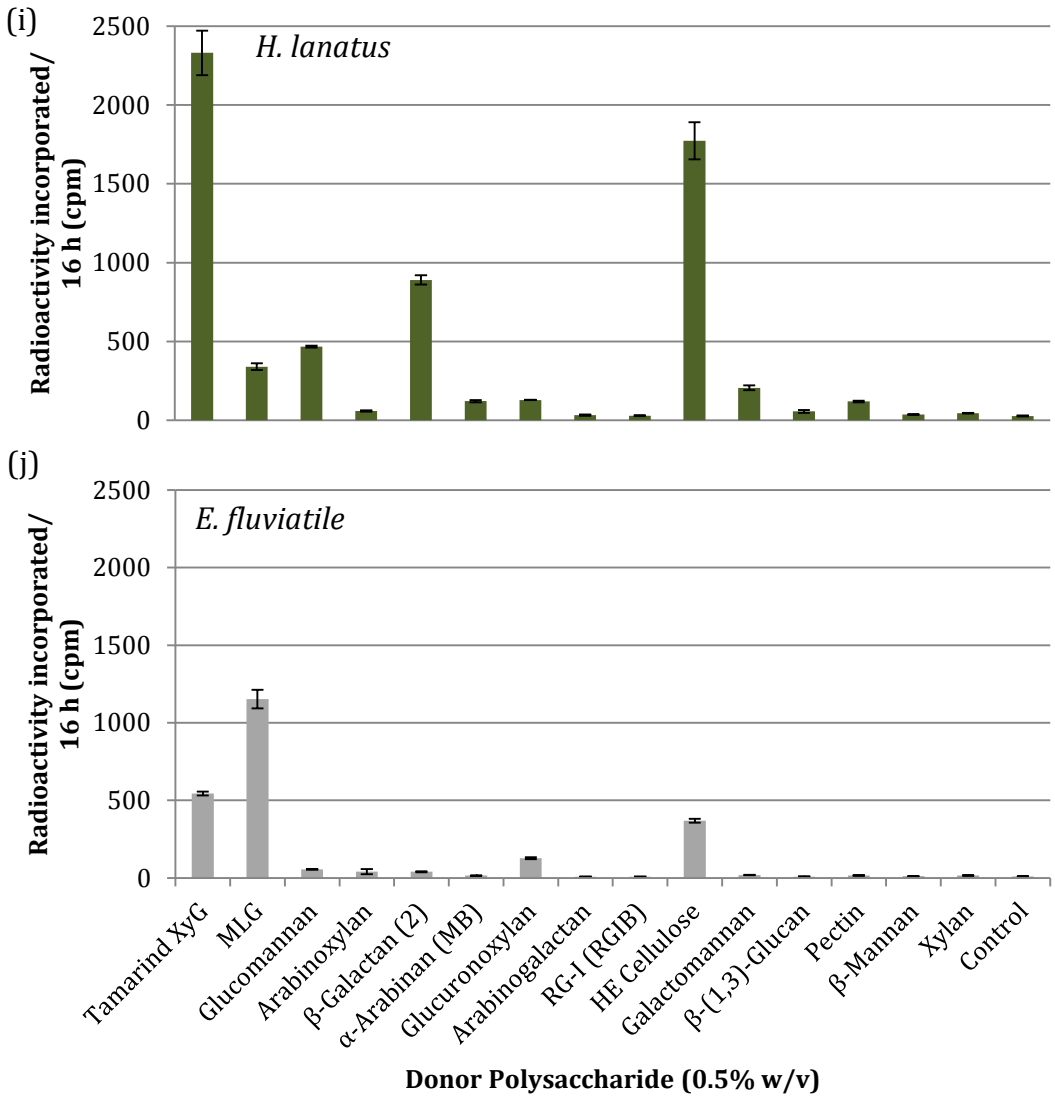


Figure 33: Broad-spectrum radio-assay for novel transglycanase activity using enzyme extracts from a range of plant sources
Enzyme extracts from (a) snowdrop, (b) *E. arvense*, (c) beansprout, (d) Arabidopsis, (e) asparagus, (f) cauliflower, (g) tomato (skin and outer pericarp), (h) crocus, (i) *H. lanatus*, (j) *E. fluviatile*, (k) *M. polymorpha*, (l) chicory and (m) enzyme-free control were tested for novel transglycanase activities with a [^{3}H]XXXGol (1 kBq) acceptor and a range of potential donor polysaccharides (0.5% w/v) for 16 h at room temperature. Error bars show standard error from triplicate results.


(c)
Beansprout
Radioactivity incorporated/ 16 h (cpm)
10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
(d)
Arabidopsis
Radioactivity incorporated/ 16 h (cpm)
(e)
Asparagus
Radioactivity incorporated/ 16 h (cpm)
Tamarind XyG
MLG
Glucomannan
Arabinoxylan
β-Galactan (2)
α-Arabinan (MB)
Glucuronoxylan
Arabinogalactan
RG-I (RGIB)
HE Cellulose
Galactomannan
β-(1,3)-Glucan
Pectin
β-Mannan
Xylan
Control
Donor Polysaccharide (0.5% w/v)


(f)
Cauliflower
Radioactivity incorporated/ 16 h (cpm)
10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
(g)
Tomato
Radioactivity incorporated/ 16 h (cpm)
(h)
Crocus
Radioactivity incorporated/ 16 h (cpm)
Tamarind XyG
MLG
Glucomannan
Arabinoxylan
β-Galactan (2)
α-Arabinan (MB)
Glucuronoxylan
Arabinogalactan
RG-I (RGIB)
HE Cellulose
Galactomannan
β-(1,3)-Glucan
Pectin
β-Mannan
Xylan
Control
Donor Polysaccharide (0.5% w/v)


(i)
H. lanatus
Radioactivity incorporated/ 16 h (cpm)
2500
2000
1500
1000
500
0
(j)
E. fluviatile
Radioactivity incorporated/ 16 h (cpm)
2500
2000
1500
1000
500
0
Tamarind XyG
MLG
Glucomannan
Arabinoxylan
β-Galactan (2)
α-Arabinan (MB)
Glucuronoxylan
Arabinogalactan
RG-I (RGIB)
HE Cellulose
Galactomannan
β-(1,3)-Glucan
Pectin
β-Mannan
Xylan
Control
Donor Polysaccharide (0.5% w/v)

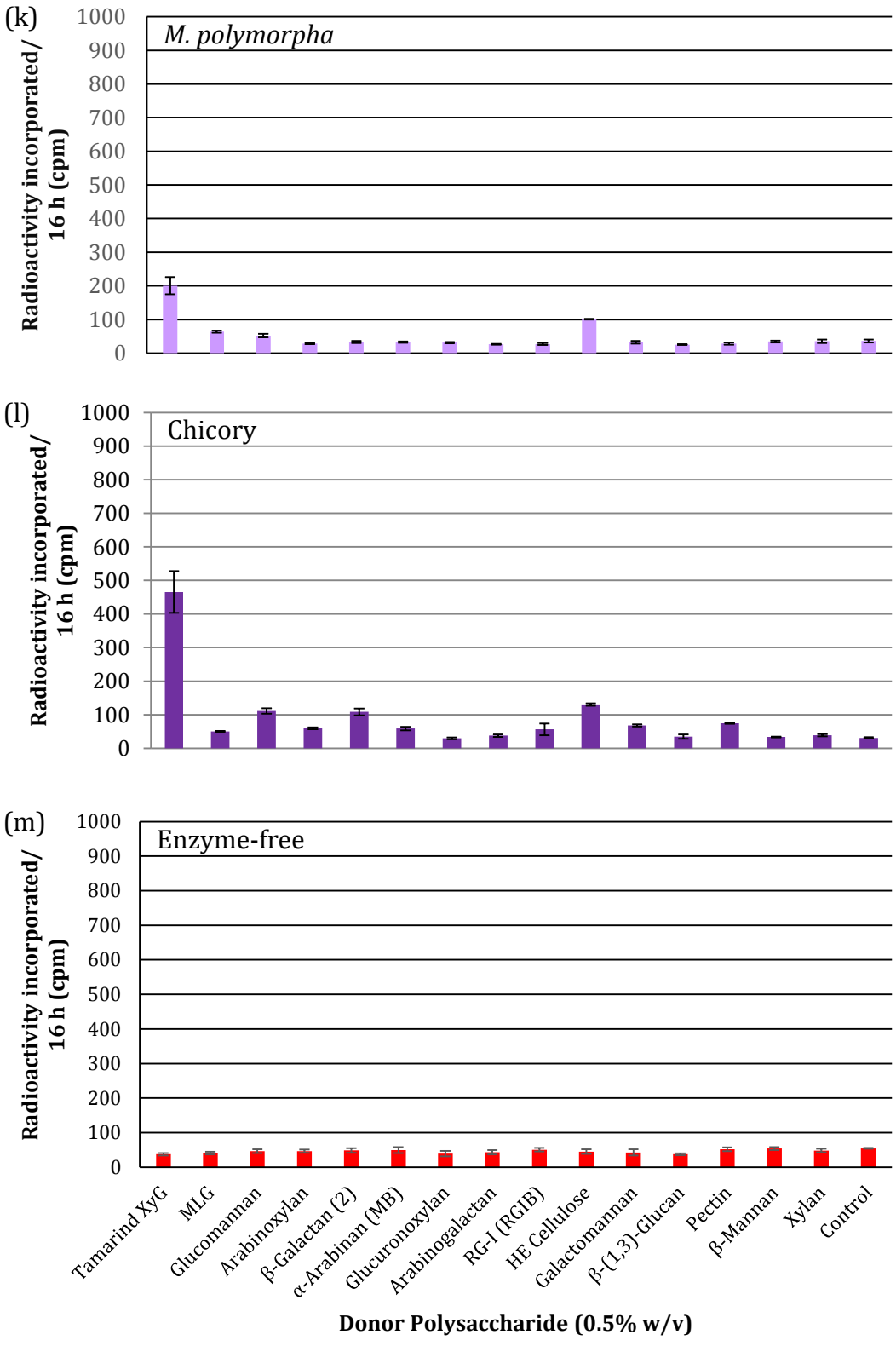

(k)
M. polymorpha
Radioactivity incorporated/ 16 h (cpm)
1000
900
800
700
600
500
400
300
200
100
0
(l)
Chicory
Radioactivity incorporated/ 16 h (cpm)
(m)
Enzyme-free
Radioactivity incorporated/ 16 h (cpm)
Tamarind XyG
MLG
Glucomannan
Arabinoxylan
β-Galactan (2)
α-Arabinan (MB)
Glucuronoxylan
Arabinogalactan
RG-I (RGIB)
HE Cellulose
Galactomannan
β-(1,3)-Glucan
Pectin
β-Mannan
Xylan
Control
Donor Polysaccharide (0.5% w/v)

Table 4: Summary of transglycanase activity observed with a range of donor polysaccharides and enzyme extracts. (1) Tx, (2) MLG, (3) Glucomannan, (4) Arabinoxylan, (5) βG 2, (6) αA MB, (7) Glucuronoxylan, (8) AG (Sigma), (9) RG-IB, (10) HEC, (11) Galactomannan, (12) β-(1,3)-Glucan, (13) Pectin, (14) β-Mannan, (15) Xylan, (16) Donor-free control. All transglycanase reactions were incubated for 16 h at room temperature as described in 2.7.4. Level of transglycanase activity rated on a qualitative scale from (+) above background to (++++) high transglycanase activity

Enzyme extract	Radioactivity incorporated (cpm) with donor polysaccharide (0.5% w/v) number:															
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Arabidopsis	5534	626	1330	369	3196	298	235	214	80	2808	812	251	928	284	284	264
	++++	+	++	+	+++					+++	++		++			
Asparagus	9719	2325	2230	1240	4917	815	614	737	426	7224	1469	811	1580	874	989	785
	++++	++	++	+	+++					+++	+		+	+	+	
Beansprout	9662	2120	2611	1262	5462	1172	750	1057	658	7529	2312	1074	2408	1326	1118	855
	++++	++	++	+	+++	+		+		+++	++	+	++	+	+	
Cauliflower	3317	1117	1172	1181	1765	941	533	1254	837	2900	783	1377	921	1008	713	845
	++++	+	+	+	++	+		+		+++		++	+	+		
Chicory	466	50	111	60	108	59	30	38	56	130	68	35	74	34	39	31
	++++		++	+	++	+				++	+		+			
Crocus	6620	1199	1035	292	1610	453	281	171	152	4420	766	250	590	226	277	220
	++++	++	++		++	+				+++	+		+			
E. arvense	4768	6263	705	188	1231	712	232	96	72	3553	500	98	618	158	162	71
	+++	++++	++	+	++	++	+			+++	+		+	+	+	
E. fluviatile	545	1152	56	40	40	15	128	8	8	368	19	8	16	11	17	10
	+++	++++	+	+	+		++			++						
H. lanatus	2331	340	467	58	890	121	128	32	29	1772	205	55	119	36	44	27
	++++	++	++		+++	+	+			+++	++		+			
M. polymorpha	200	64	52	29	33	33	31	26	27	101	32	25	28	34	35	36
	++++	+	+							++						
Snowdrop	7668	1564	1489	473	2103	780	401	281	160	5469	883	319	1004	374	405	355
	++++	++	++	+	++	+	+			+++	+		++		+	
Tomato	6774	1147	1192	891	3169	841	170	1087	400	3241	1388	690	932	965	907	142
	++++	++	++	+	+++	+		+	+	+++	++	+	+	+	+	
Control	37	41	47	46	49	49	39	43	50	45	43	37	52	54	48	54

6 Results: Identification of the MXE gene from *Equisetum fluviatile*

It has been hypothesised in the literature (Fry *et al*., 2008; Sørensen *et al*., 2008) that *Equisetum* XET and MXE activities are catalysed by different transglucanases due to significant differences in the characteristics of XET and MXE activity and their expression patterns (e.g. XET expression peaks in growing tissue while MXE expression is highest in older tissues). A central aim of this project was to identify and heterologously express the gene (or genes) which encode MXE.

MXE from a crude *E. fluviatile* extract, from which high levels of MXE activity had previously been detected, was purified by Dr. Tom Simmons via step-wise processing of MXE-active fractions by ammonium sulphate precipitaton, SEC (Biogel P100), affinity chromatography (Conconavalin A) and isoelectric focussing. MXE was found to have a molecular weight of ~30 kDa and a pI ~4. The purified MXE protein sample was run on an SDS-PAGE and stained with Coomassie Blue. A distinct band (MW = ~30 kDa) was extracted and underwent trypsin digestion and sonification to produce digestion products for MALDI-TOF and LC-MS.

The processed spectra (KE Mohler & TJ Simmons, unpublished date) were used to conduct a BLAST search of the NCBI non-redundant database and an *E. fluviatile* transcriptome database using MASCOT software. From these gene fragments, 11 genes were identified with sequence homology to known XTHs. Having identified candidate MXE genes, primers were designed to allow amplification of the putative mature protein (without the signal peptide) from *E. fluviatile* cDNA.

Infusion PCR cloning and transformation of *Escherichia coli* and *Pichia pastoris* yielded 9 constructs (five GH16s, three GH17s and one GH64) with methanol-induced expression leading to secretion of the desired protein into the culture media. Each recombinant protein was assayed for transglucanase activities to investigate whether any had a comparable XET : MXE : CXE ratio to a purified *E. fluviatile* MXE extract (pMXE) thus indicating an *Equisetum* MXE-encoding gene.

6.1 Transglucanase activity of crude *Equisetum fluviatile* and purified MXE extracts provide a representative XET : MXE : CXE ratio

Purified MXE (pMXE), courtesy of Dr Tom Simmons, and crude late season *E. fluviatile* enzyme extracts, in which significant MXE and CXE activity had previously been detected, were assayed for XET, MXE and CXE activity to act as positive controls. The activity observed with pMXE provided a representative ratio of XET : MXE : CXE activity for MXE, allowing comparison between this and that observed following expression of the candidate MXE genes in *P. pastoris*. The XET : MXE : CXE ratio was approximately 1 : 2.8 : 1.5 (Figure 35f) and 1 : 1.25 : 0.8 (TJ Simmons, unpublished data) for pMXE and the crude *Equisetum* extracts respectively. Higher relative XET activity in the crude *Equisetum* extract is potentially due to the presence of endogenous Equisetum XTHs that are absent in pMXE.

6.2 GH16 constructs

All members of the GH16 family of enzymes tested (constructs 377, 383, 385, 397 and 398) exhibited significant XET activity (Figure 35a-e). MXE and CXE activity were markedly lower and varied relative to XET between candidate recombinant MXE proteins. A trend of higher MXE activity correlating with higher CXE activity was observed by us, supporting the hypothesis that MXE and CXE activities are catalysed by the same, predominately MXE, protein.

Multiple sequence alignment of all heterologously expressed GH16 XTH genes (Figure 34) shows high conservation of primary structure between constructs with sequence identity of 61.7-99.6% to construct 383. Construct 383 was deemed the consensus candiate MXE gene as it shared the highest homology with the partial gene sequences determined from MS-data. The primary structure of constructs 397 and 398 differed from that of construct 383 by one amino acid, and thus differed from each other by two amino acids. Construct 397 had an S to P substitution at position 283 while 398 had an L to A substitution at position 118. As expected, due to the propensity for a conserved binding cleft and active site topology within a GH family, sequence homology was higher in regions

flanking the conserved active site (ELDFE), including the *N*-glycosylation sites 3-15 residues downstream of this which have been proposed to be integral to XET function (Campbell and Braam, 1999a; Henriksson *et al.*, 2003; Johansson *et al.*, 2004).

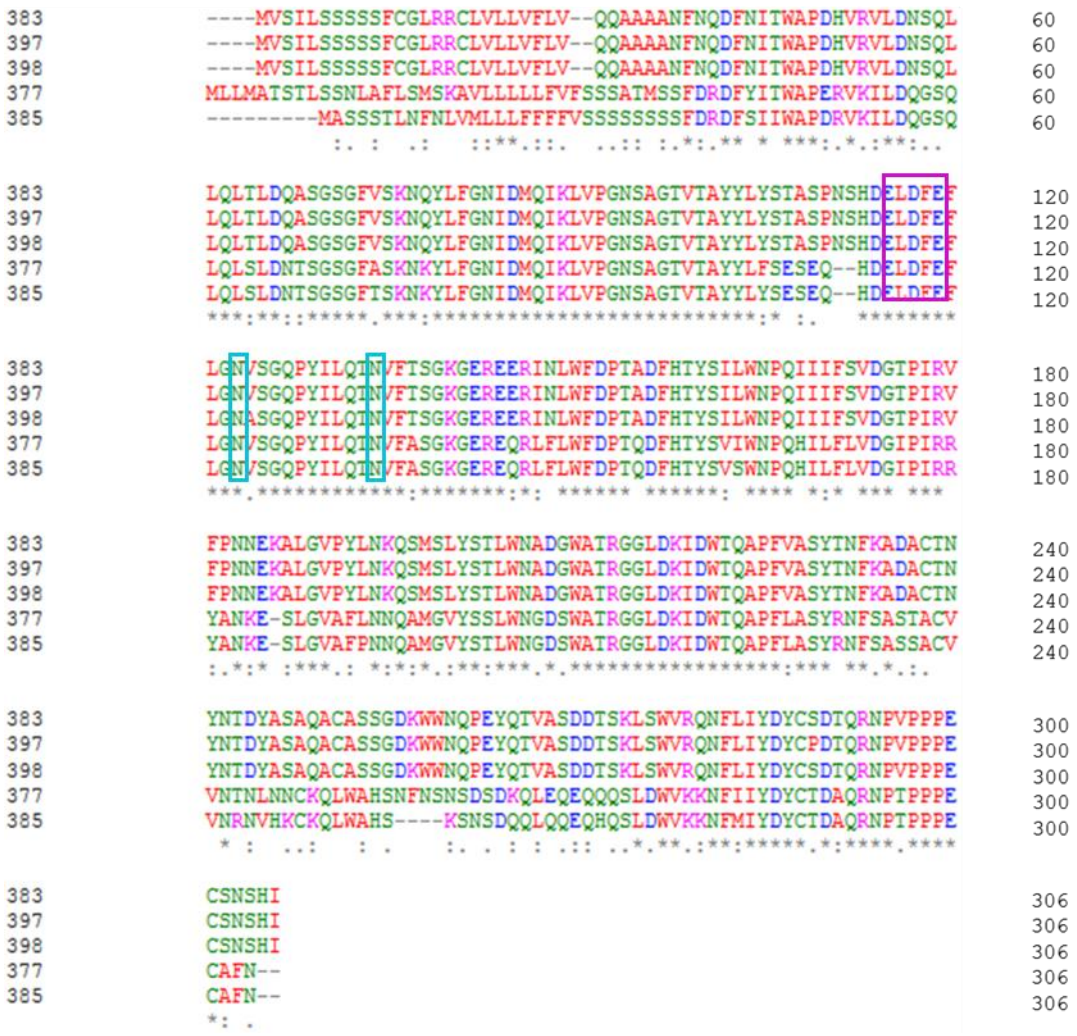


Figure 34: Sequence alignment of GH16 constructs
ClustalW multiple sequence alignment by MUSCLE (3.8) indicating hydroxyl/sulfhydryl/amine/glycine (**green**), small and hydrophobic (**red**), acidic (**blue**) and basic (**pink**) amino acids. Homology between constructs is indicated as (*) identical, (:) conserved substitutions, and (.) semi-conserved substitutions. The active site residues (**pink box**) and the conserved N-glycosylation sites (**cyan box**) are highlighted.

6.2.1 GH16 recombinant proteins are highly XET-active XTHs with MXE and CXE activities varying between constructs

Enzyme 383 displayed both the second highest MXE and highest CXE activity, approximately 3.76% and 8.14% of the XET activity respectively (Figure 35a). Similar XET : MXE : CXE ratios were observed for enzymes 398 and 385. Enzyme 398 produced MXE activity whch was 2.46% of that observed for XET and CXE which was 3.69% of XET (Figure 35c). Meanwhile, 385 produced MXE activity which ws 2.86% of XET, slightly higher than detected in 398, but CXE activity of 2.07% which was slightly lower than with 398 (Figure 35e).

Although 397 indicated the highest CXE and second highest MXE activity, 11.4% and 5.7% of XET respectively (Figure 35b), the actual quantity of radioactivity incorporated was extremely low compared to other constructs. For example, radioactivity incorporated during the XET assay on 383 was 3515 cpm compared to just 35 cpm for 397. The low level of XET activity observed indicates that the 397 extract is not very active, most likely due to poor expression resulting in either a low enzyme concentration or an inactive enzyme due to faults in post-translational modifications. Thus, results for 397 are unreliable and should not be considered a true representation of XET : MXE : CXE activity.

The highest XET activity of all GH16 recombinant proteins was observed with 377. Interestingly, despite 377's XET activity almost doubling that of 383, its MXE activity was significantly lower (Figure 35d). A similar trend was observed for CXE activity. Expressed as a percentage, MXE activity of 377 is 0.17% of XET activity whilst CXE activity is 0.33% of XET. The high level of XET activity and low degree of side-reactions occurring relative to other enzyme indicates that 377 is a more specific XET than other constructs tested.

As explained in 2.8.7.1 and 2.8.8, the donor polysaccharide for XET and MXE assays is an aqueous-soluble XyG or MLG as opposed to the alkali-treated paper, which is a source of cellulose, in the CXE assay. The difference in substrate solubility influences the availability of donor polysaccharides to the enzyme and the detection of transglucanase products. In addition, a greater volume of enzyme

was used for the CXE assays (33 µl) compared with the XET and MXE assays (10 µl) and, as such, an exact concentration of cellulose donor polysaccharide can not be calculated unlike XyG and MLG. Inconsistencies between assays mean that no direct comparisons can be made between the XET or MXE assays and the CXE with respect to relative radioactivity incorporated. However, by expressing activity per given volume for each transglucanase activity, it is possible to calculate a ratio of all three activities and compare this to the ratio of activities observed with pMXE. As a result, although we cannot determine how much of XET or MXE activity CXE correlated to, we can still aim to recognise the desired MXE-active protein.

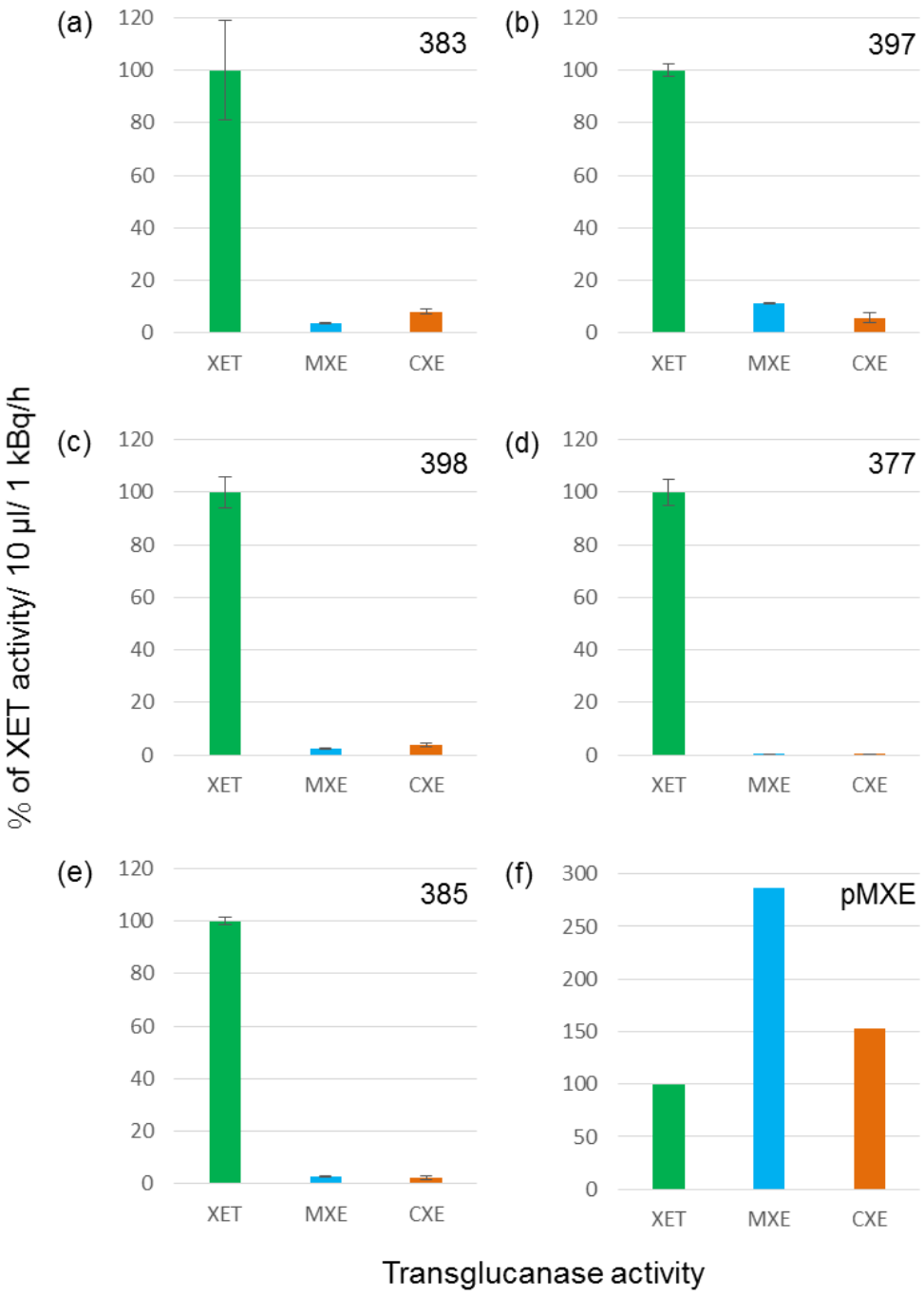


Figure 35: XET, MXE and CXE activity GH16 candidate genes
Transglycanase activities of (a) 383 (b) 397 (c) 398 (d) 377 (e) 385 and (f) pMXE showing radioactivity incorporated during 1 h incubation with 10 µl enzyme extract. All results are corrected for enzyme-free controls. A 1-h incubation time was selected to ensure maximum incorporation of radioactivity was not achieved by XET allowing an estimate of XET : MXE : CXE ratio. All transglycanase assays for all enzymes, with the exception of pMXE, were carried out in triplicate. Results presented for pMXE are consistent with the ratios previously observed for this enzyme. Error bars indicate standard error of triplicate results.

The XET : MXE : CXE ratios of all GH16 recombinant XTHs are shown in Table 5. All GH16 XTHs are highly XET-active XTHs with enzyme 377 having the highest specificity for this activity. No observed XET : MXE : CXE ratios are comparable to that of pMXE. However, there is a clear correlation between MXE and CXE activity with both activities detectable at low levels.

Table 5: Summary of GH16 recombinant protein XET, MXE and CXE activities
Data were averaged from results of assays conducted in triplicate and corrected for enzyme-free controls.

Construct	XET/ 10 µl/ h (cpm)	MXE/ 10 µl/ h (cpm)	CXE/ 10 µl/ h (cpm)	XET: MXE: CXE
383	3513	132	286	100: 3.76: 8.14
397	35	4	2	100: 11.4: 5.70
398	407	10	15	100: 2.46: 3.69
377	5835	10	19	100: 0.17: 0.33
385	629	18	13	100: 2.86: 2.07

Further analysis was needed to confirm that the XET: MXE: CXE ratio observed for these GH16 constructs was a true representation of relative transglucanase activities rather than a result of, for example, a lack of required post-translational modifications or inhibition by an endogenous *P. pastoris* protein (see sections 6.8 and 6.9).

6.3 GH17 Constructs

Integration of the GH17 XTH-like genes (379, 399, 400) (Figure 36) into the pPICZαA plasmid and transformation of *P. pastoris* was successful, as confirmed by dot-blot analysis. However, secretion of GH17s proved difficult with only low concentrations of *myc*-tagged protein being detected via dot-blot following expression in BMMY. To optimise expression conditions for the GH17 constructs a range of different expression media (see 2.8.3) and incubation temperatures were tested. Construct 379 allowed secretion of protein into MM medium at a high enough concentration for activity assays to be conducted. However, 399 and 400 only secreted *myc*-tagged proteins at a very low concentration into BMM medium and due to time constraints this method could not be improved upon. As

a result, no further analysis was possible for constructs 399 and 400. All GH17 constructs preferred an incubation temperature of 30°C for expression as opposed 37°C, which was favourable for GH16 constructs.

Figure 36: Sequence alignment of GH17 genes
ClustalW multiple sequence alignment by MUSCLE (3.8) of GH17 genes showed high sequence homology between constructs. Colour coding as described in Figure 34.

Enzyme 379 showed extremely low levels of XET (5 cpm), MXE (4 cpm) and CXE (1 cpm) activity per 10 µl enzyme extract for 1 h incubation. Extension of the incubation time to 16 h still resulted in XET (25 cpm), MXE (12 cpm) and CXE (6 cpm) activity that could be considered negligible when compared to the enzyme free controls (17 cpm, 16 cpm and 21 cpm for XET, MXE and CXE respectively).

6.4 GH64

Enzyme 381 (Figure 37) was the only GH64 enzyme tested for transglucanase activity and showed no significant XET, MXE or CXE activity, with radioactivity detected only marginally elevated compared to background, possibly attributed to impurities in the *Pichia* secetions (see 6.6 and 6.7). Due to the lack of activity

observed, further analysis of this family was not deemed a priority and the other GH64 selected (401) was not used for transformation of *P. pastoris*.

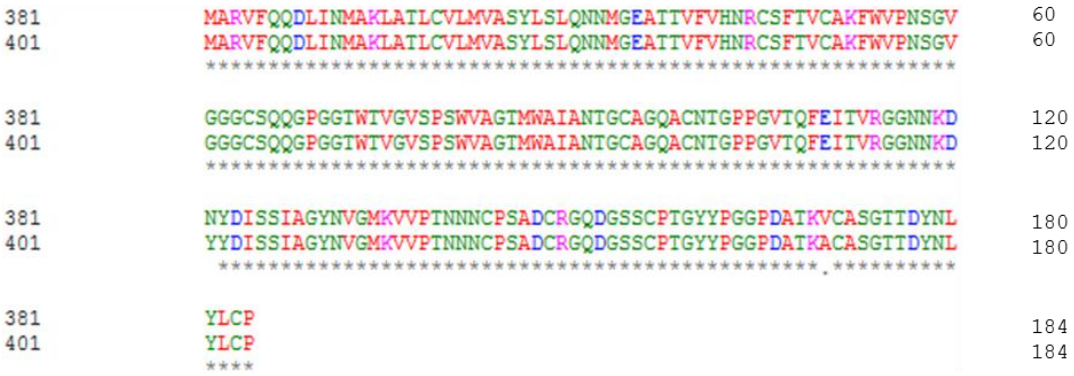


Figure 37: Sequence alignment of GH64 constructs
GH64 gene sequences were aligned in the format of ClustalW multiple sequence alignment by MUSCLE (3.8). Sequence identity was high between GH64 constructs.

Following preliminary investigations into the XET : MXE : CXE ratio for all *Pichia* expressed proteins, only GH16 enzymes displayed significant levels of transglucanase activity although the XET : MXE : CXE ratio did not correlate with that observed for pMXE. Further analysis was conducted on the most likely MXE candidate, construct 383, to determine the initial rate of reaction and thus a more accurate ratio of XET : MXE : CXE activity, ensure post-translational modifications had been conducted and to investigate whether there was any inhibition of MXE activity by endogenous *Pichia* secreted proteins.

6.5 Timescales of XET, MXE and CXE activity of 383 to determine initial rate

Due to the highly active nature of the GH16 recombinant XTHs as XETs, the 1-h incubation period used previously may have been too long, resulting in incorporation of different quantities of the total radioactivity for each transglucanase which would influence their rate of reaction. This would mean

that the ratios of XET : MXE : CXE products observed may not have been a true representation of relative activities.

I conducted tranglucanase assays at a multitude of timepoints across a 24-h timescale to determine the initial reaction rate which allowed (a) an accurate XET : MXE : CXE to be calculated and (b) observation of activity progression over this time period. We assayed both unpurified (Figure 38) and IMAC (see 2.4.5) purified recombinant 383 (Figure 39) for XET, MXE and CXE activity. A timescale of pMXE transglucanase activity was also conducted for comparison (Figure 40).

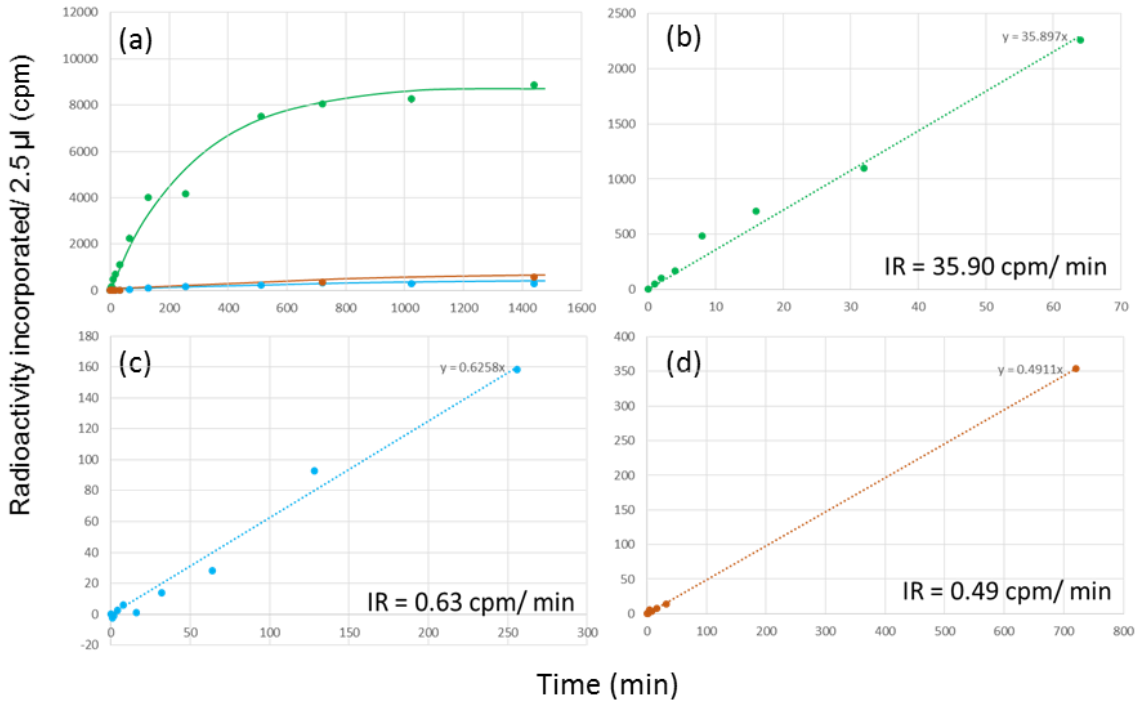


Figure 38: Initial rate for transglucanase activities and 24-h timescale for unpurified 383
(a) Full 24-h timescale of XET (**green**), MXE (**blue**) and CXE (**orange**) activities of unpurified 383 assayed for radioactivity incorporated per 10 µl enzyme extract. Also shown are the sections of linear initial activity for (b) XET (c) MXE and (d) CXE from which the initial rate of reaction was calculated. IR = initial rate.

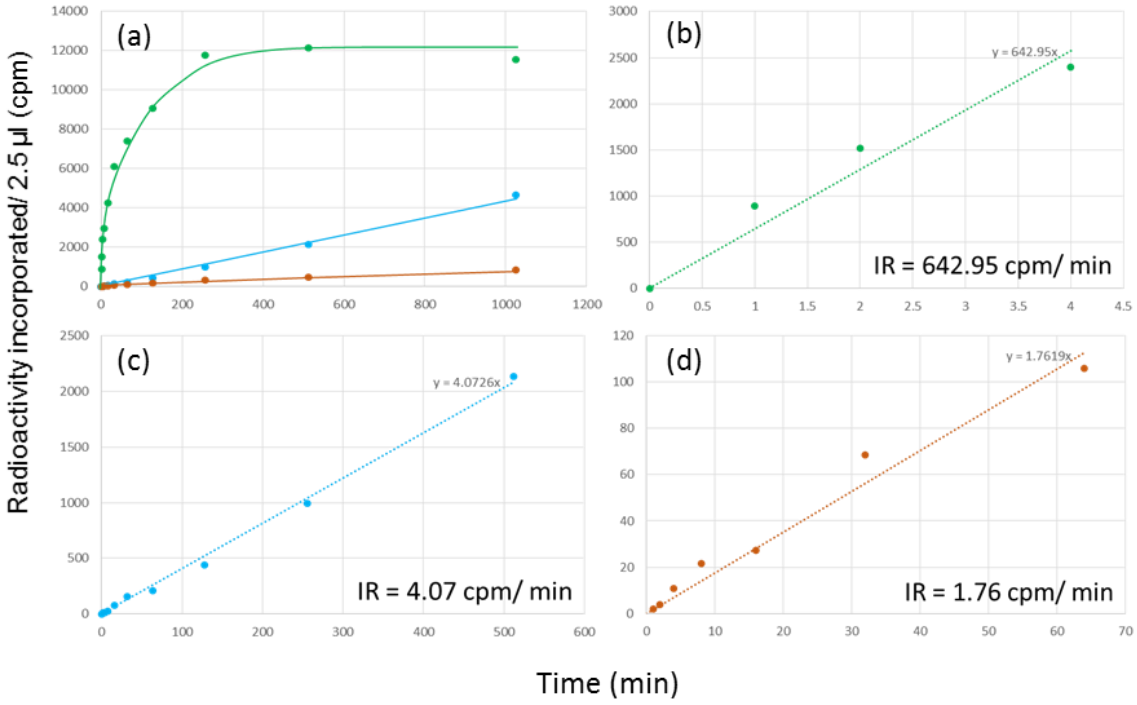


Figure 39: Initial rate for transglucanase activities and 24 h timescale for His-tag purified 383
(a) Full 24-h timescale of XET (**green**), MXE (**blue**) and CXE (**orange**) activities of purified 383. Linear initial activity for (b) XET (c) MXE and (d) CXE were used to determine the initial rate of reaction for each activity.

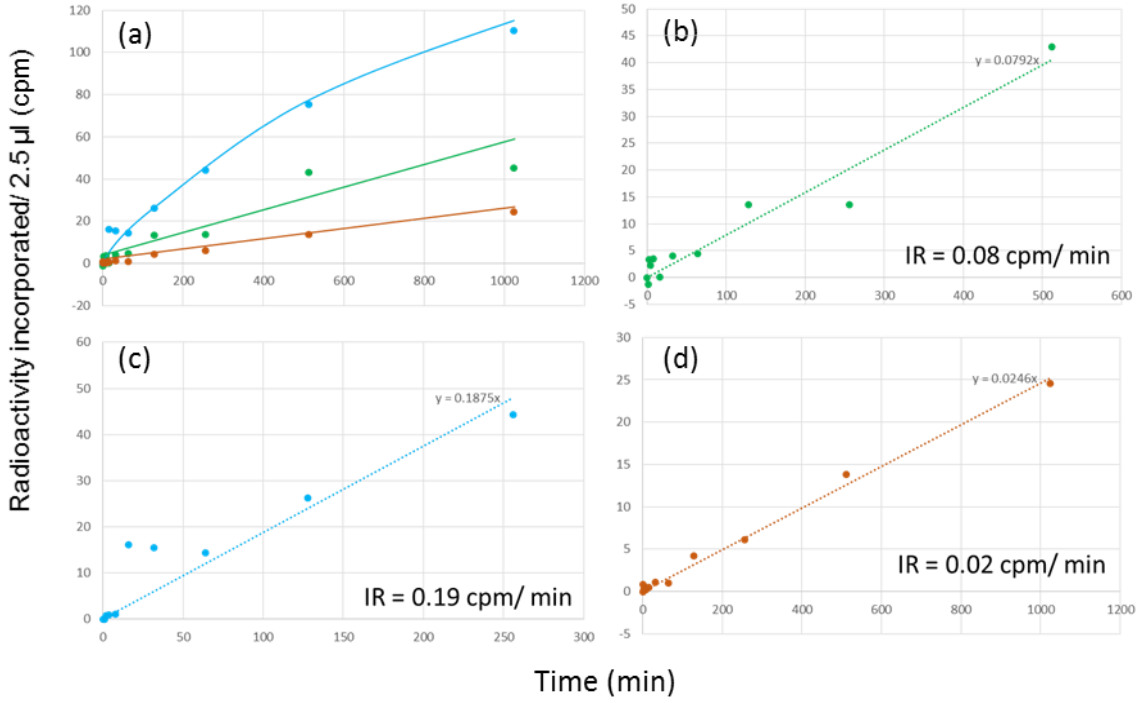


Figure 40: Initial rate for transglucanase activities and 24-h timescale for purified MXE from a crude *Equisetum* extract
(a) Full 24-h timescale of XET (**green**), MXE (**blue**) and CXE (**orange**) activities of pMXE as well as linear initial activity for (b) XET (c) MXE and (d) CXE which were used to determine the initial rate of reaction for each activity.

Unpurified 383, as used in the previous experiments, gave high XET activity but the yield of transglucanase products plateaued after approximately 800 min as it reached the maximum incorporation of radioactivity which was limited by available acceptor substrate (Figure 38). If higher concentrations of acceptor substrate had been available the amount of XET activity would have continued to increase. The MXE and CXE activity observed for unpurified 383 were much lower than the XET activity.

Interestingly, MXE activity was limited to a maximum incorporation of around 300 cpm regardless of the XET activity (Figure 38). Despite an initial rise, the yield of MXE product plateaued. It would be expected that if, as proposed, MXE activity was a side reaction of XET then the ratio of XET to MXE activity would remain constant throughout. As XET activity was only limited by the amount of donor and acceptor substrates available, unpurified 383 should still have been

able to conduct MXE activity even when XET activity had plateaued. Hence, it would be expected that the quantity of MXE product would continue to increase. This did not happen, indicating potential inhibition of MXE activity most likely due to shielding or degradation of the donor polysaccharide by a component of the endogenous *Pichia* secretions. It could be suggested that limiting of MXE activity was indicative of enzyme inhibition. However, this seems unlikely as, if the enzyme was inhibited, we would expect to observe an effect on the XET activity too, which we did not. Theoretically, if MXE activity was catalysed by a second active site in the recombinant XTH then inhibition of the protein could occur without necessarily affecting XET activity. However, a second active site has never been reported as a possibility with any GH16 enzymes and it seems unlikely, due to the lack of domains with a high density of negative residues which interact with aromatic residues within the binding cleft to act as a second active site. Another caveat with this theory is that the binding of a substrate to one active site would feasibly incur a change in the overall structure of the enzyme, altering the access and function of another active site which would also be detected by changes in XET activity. Further analysis was required to confirm the cause of the MXE inhibition.

The timescale obtained from transglucanase assays of His-tag purified 383 (p383) supports the theory that MXE activity is limited due to inhibition of the donor polysaccharides by something within the natural *Pichia* secretion (Figure 39). The yield of MXE product of p383 continues to increase even after the yield of XET product has plateaued and in excess of 300 cpm. The XET activity of p383 has a significantly higher initial rate than the XET activity of unpurified 383, 35.9 cpm/ min compared to 642.95 cpm/ min for unpurified 383 and p383 respectively.

The ratio of XET : MXE : CXE, based on the initial rate of reaction for each transglucanase activity, for unpurified 383 is 100 : 1.75 : 1.36 (Figure 38), lower than that previously stated (Table 5). p383 presented a ratio of 100 : 0.63 : 0.27 (Figure 39), displaying a lower proportion of MXE activity than unpurified 383, probably due to the dramatically increased initial rate of XET activity. pMXE gave

a ratio of 100 : 237.5 : 25 (Figure 40) with MXE activity well in excess of that observed for either 383 samples.

The CXE activity observed for pMXE (Figure 40) and unpurified 383 (Figure 38), is much lower than previously observed (Figure 35). This could be due to the use of a much lower amount of stock enzyme – 8.25 µl rather than 33 µl - than previously used. It is possible that the lower amount of enzyme was not able to properly infiltrate the paper which is required for transglucanase activity, although it was scaled down proportionally.

6.6 Incubation of MLG with endogenous *Pichia pastoris* secretions leads to a dramatic decrease in viscosity

As a method of determining whether the limitation of MXE activity observed was due to a physical change of MLG by the *Pichia* endogenous proteins, I conducted a viscosity assay. The aim of this experiment was to determine whether the viscosity of MLG was altered following incubation with natural *Pichia* secretion product, indicating a change in the net length of MLG molecules within the solution.

The results showed a notable decrease in viscosity following 12-h incubation with the *Pichia* extract (Table 6). The loss of viscosity indicates a shorter net length of MLG molecules resulting from degredation. This suggests that MXE activity could have been limited either because donor polysaccharides were too small for transglucosylation to occur or the transglucanase products were of insufficient MW to remain bound to paper during washings and were consequently undetected.

Table 6: Viscosity assay to determine effects of endogenous *Pichia* proteins on MLG

0.4% MLG (200 µl) was incubated for 12 h with either endogenous *Pichia* secretion products (pPICZαA empty vector; 20 µl) or an equal volume of MES buffer (50 mM, pH 6.0) as a control. dH_2O was tested to act as an indicator of run time expected following complete loss of viscosity. SE indicates standard error from 5 repeats.

Solution	Run Time (*s*) ± SE
0.4% MLG Control	95.0 ± 2.0
0.4% MLG + pPICZαA	5.8 ± 0.2
dH_2O	≤1

6.7 Mixing of pMXE with natural *Pichia* secretions results in a significant loss of MXE activity

Mixing assays were conducted in addition to viscosity assays to determine whether the natural *Pichia* secretions inhibit MXE activity. pMXE was assayed for XET and MXE activity with and without the secretions of *Pichia pastoris* which expressed the empty pPICZαA plasmid (referred to from now on as pPICZαA).

The MXE activity of pMXE observed following incubation with pPICZαA was reduced to almost 50% of the activity observed with pMXE alone (Figure 41). Interestingly, the effect on XET activity was much less, further supporting the theory that MLG itself is being degraded rather than the enzyme being inhibited. pPICZαA itself lacked XET or MXE activity.

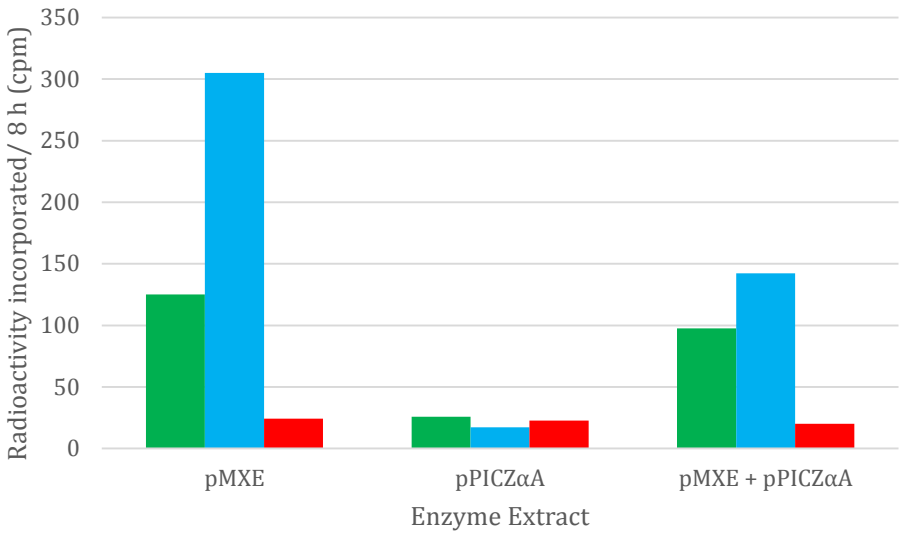


Figure 41: Transglucanase assay to determine the effect of *Pichia* secretions on pMXE XET and MXE activity
XET (**green**) and MXE (**blue**) activity were assayed for pMXE samples, with and without *Pichia* secretions, and pPICZαA. A donor-free control (**red**) was also included to detect any endogenous enzymes within Pichia secretions capable of transglucanase activities.

As viscosity and mixing experiments indicated the potential degredation of MLG by components of the natural *Pichia* secretions, I decided to use purified samples of 383, and all other *Pichia*-expressed recombinant proteins, for all future assays to allow proper detection of MXE activity.

6.8 Deglycosylation leads to a dramatic loss in both XET and MXE activity

Glycosylation is a post-translational modification leading to the formation of a glycoprotein by the addition of a glycosyl group to an arginine, asparagine, cysteine, serine, threonine, tyrosine, tryptophan, or hydroxylysine residue. To investigate (a) whether this post-translational modification was processed following expression of our recombinant XTHs and (b) the effect of deglycosylation on XET and MXE activity we incubated purified recombinant proteins 383 and 398 with EndoH (see 2.8.9.2) which removes oligosaccharide sidechains from the the polypeptide chain.

Deglycosylation resulted in a dramatic decrease in XET activity but the extent of this varied between the recombinant XTHs tested (Figure 42). Although both p398 and p383 lost significant XET activity, p383 activity only decreased to around 50% of the EndoH-free control compared to approximately 30% for p398. This supports the hypothesis for a conserved glycosylation site for XET activity (Campbell and Braam, 1999a; Henriksson *et al*., 2003; Johansson *et al*., 2004) but suggests that, although glycosylation at this site supports XET activity it is not an absolute requirement. MXE activity for both XTHs significantly decreased, almost to background, comparable to the trend observed with XET for these enzymes. This indicates that the MXE activity observed for these recombinant proteins is a side reaction of XET.

Deglycosylation of pMXE led to a dramatic decrease in MXE activity to levels just above background following incubation with EndoH. This indicates a requirement, in excess of that of the XTHs tested here, for glycosylation of the MXE protein for activity. It would be interesting to determine which residue is responsible for this but due to time constraints we were unable to.

Figure 42: Deglycosylation of p383, p398 and pMXE by EndoH
Enzyme extracts (a) p383 (b) p398 (c) pMXE were incubated for up to 24 h with EndoH and then used in XET (**green**) and MXE (**blue**) transglucanase assays.

6.9 Dephosphorylation significantly inhibits MXE but not XET activity

Phosphorylation is the addition of a phosphate group to a protein or other organic molecule which can lead to the activation or deactivation of many enzymes. Phosphorylation usually occurs on the serine (S), threonine (T), tyrosine (Y) and histidine residues (H). To investigate the importance of this post-translational modification for the function of my recombinant XTHs I dephosphorylated them with an acid phosphatase from *E. coli* (see 2.8.9.1). It is thought that this phosphatase dephosphorylates residues progressively from the N- to the C-terminal.

I conducted a timescale of dephosphosylation, testing samples at 2-h intervals to assay the effect on transglucanase activity. I dephosphorylated p383 which was

the most MXE-active of our recombinant XTHs, p398 which was a highly active XET, and pMXE (Figure 43). Dephosphorylation of p383 caused a decrease in activity by approximately 50% between 0 and 2 h and again by another 80% between 4 and 6 h before dropping to background levels of activity between 6 and 8 h. Unexpectedly, activity increased again between 16 and 18h. As these results were from a single sample rather than replicates, due to limited purified recombinants, the reliability of these results is questionable meaning the fluctuations in activity observed here may not be representative of the true effect of dephosphorylation on this protein. To establish this, the assay needs to be repeated. However, if this fluctuation in activity is real then it could indicate dephosphorylation of a residue which requires a phosphate group for XET activity which can be rescued by dephosphorylation of another site downstream from this. Although this seems unlikely as phosphorylation did not have any real effect on the activity of the highly active XET p398, if the trend observed with p383 was real it would be due to the presence of a residue that is not conserved throughout XTHs.

pMXE showed a significant initial drop in MXE activity between 6 and 8 h following dephosphorylation. After a plateau the MXE activity decreased again between 20 and 22 h of dephosphorylation, almost to background levels. This trend was not mirrored for XET activity thus indicating a requirement for phosphorylation for MXE activity. The double decline pattern observed here could indicate the presence of two distinct residues, located far apart in the primary sequence of the protein, which require phosphorylation for MXE activity to be viable.

Although this dephosphorylation assay was not reproduced and we were unable to identify sites of phosphorylation, it was a quick and convenient method which suggested that post-translational phosphorylation had probably occurred.

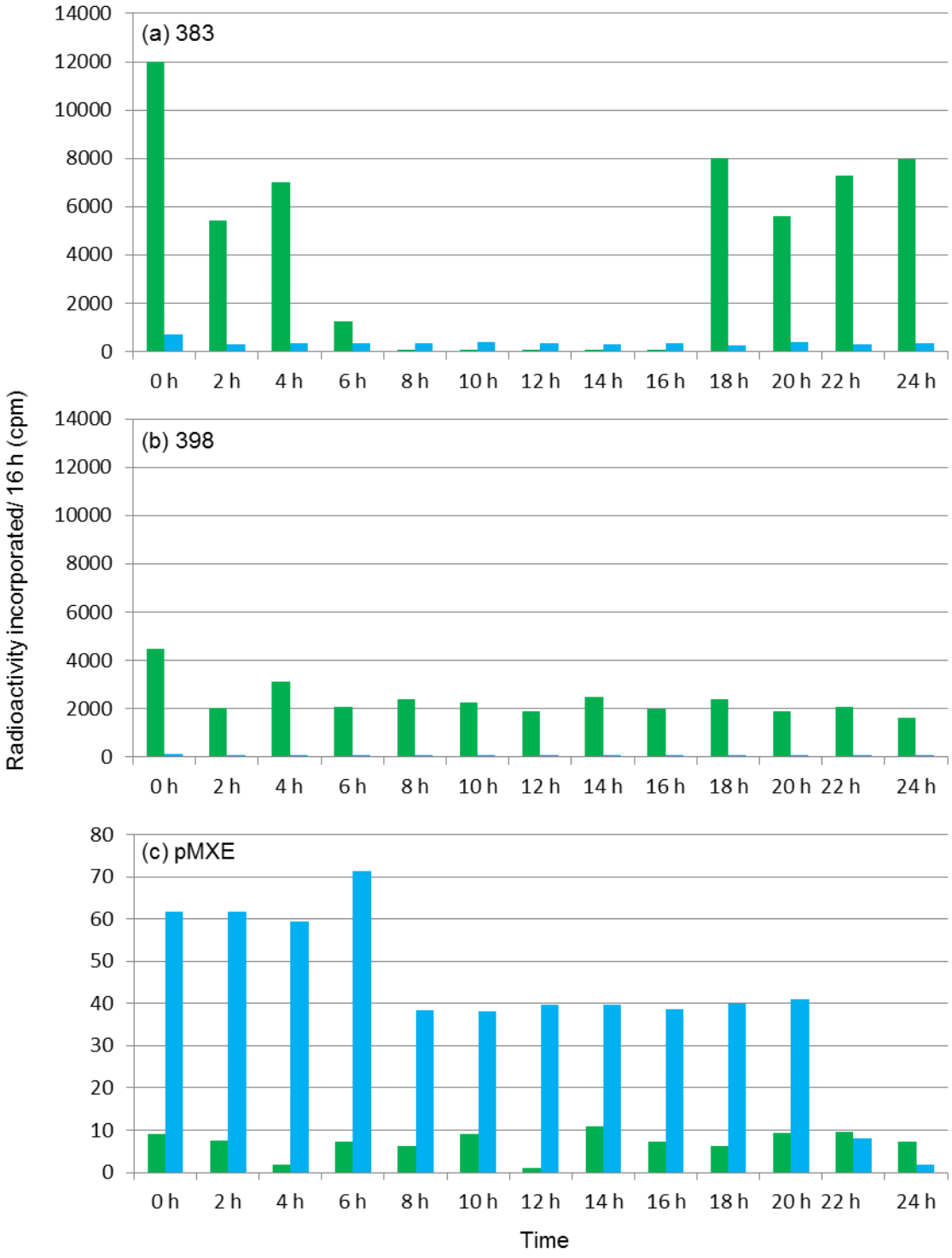


Figure 43: Dephosphorylation timescale of p383, p398 and pMXE with acid phosphatase

Enzymes (a) p383, (b) p398, and (c) pMXE were incubated for up to 24 h with acid phosphatase prior to incubation with a [^{3}H]XXXGol (1 kBq) acceptor and XyG (**green**) or MLG (**blue**) donor polysaccharide.

None of the recombinant XTHs tested from the original transcriptome produced XET : MXE : CXE ratios consistent with that of pMXE or showed the same pattern of activity following dephosphorylation and deglycosylation, strongly indicating that they are not MXE proteins. It would have been interesting to repeat this experiment to determine whether the fluctuation observed during dephosphorylation of p383 was a true representation of activity but I decided to prioritise identifying the *Equisetum* MXE gene. As such, there was insufficient time to further characterise these five new recombinant XTHs from *Equisetum*.

6.10 A new transcriptome and new candidate MXE genes

Following unsuccessful detection of the MXE gene from the previous transcriptome, a new transcriptome was sequenced for a single late-season *E. fluviatile* individual using 454 technology (Roche). The two highest scoring matches from the transcriptome database were partial gene sequences for XTH homologous proteins (courtesy of Dr Kyle Mohler and Dr Tom Simmons). However, no candidate glycan-acting proteins were identified by searching public databases. The full sequences of the 2 candidate genes were identified by the use of 5' and 3' RACE from late season *E. fluviatile* cDNA, indicating that these fragments were two parts of the same gene.

Dr. Tom Simmons is responsible for the initial work including a search of the transcriptome for candidate genes using MS data, 5' and 3' RACE, design of new primers, the production of candidate MXEs (A, C, F and G; none of which were the consensus) via high-fidelity RT-PCR and incorporation of EcoRI and XbaI restriction sites upstream and downstream respectively.

Of these 4 candidate genes, only A, C and G were successfully expressed in the heterologous *P. pastoris* expression system. Following preliminary XET and MXE assays on the secretion products of these three genes (Figure 44) and optimisation of the *Pichia* expression system (Figure 45), site-directed mutagenesis of candidate gene G was conducted by Dr. Simmons to obtain the consensus sequence.

6.11 Preliminary transglucanase assays indicate potential MXE activity of protein encoded by candidate construct G1

Preliminary results for XET and MXE activity of candidate MXE genes A, C and G from a small-scale expression assay showed low incorporation of radioactivity for both transglycanase activities (Figure 44). However, despite the low activity detected, significant MXE activity was observed in candidate gene G samples particularly G1. Multiple factors – including, but not limited to, the short incubation time, temperature, and *Pichia*-expression medium (BMMY) used – could account for the extremely low level of radioactive product produced. Further experimentation was required to optimise conditions for the expression and detection of potential MXE candidate gene G.

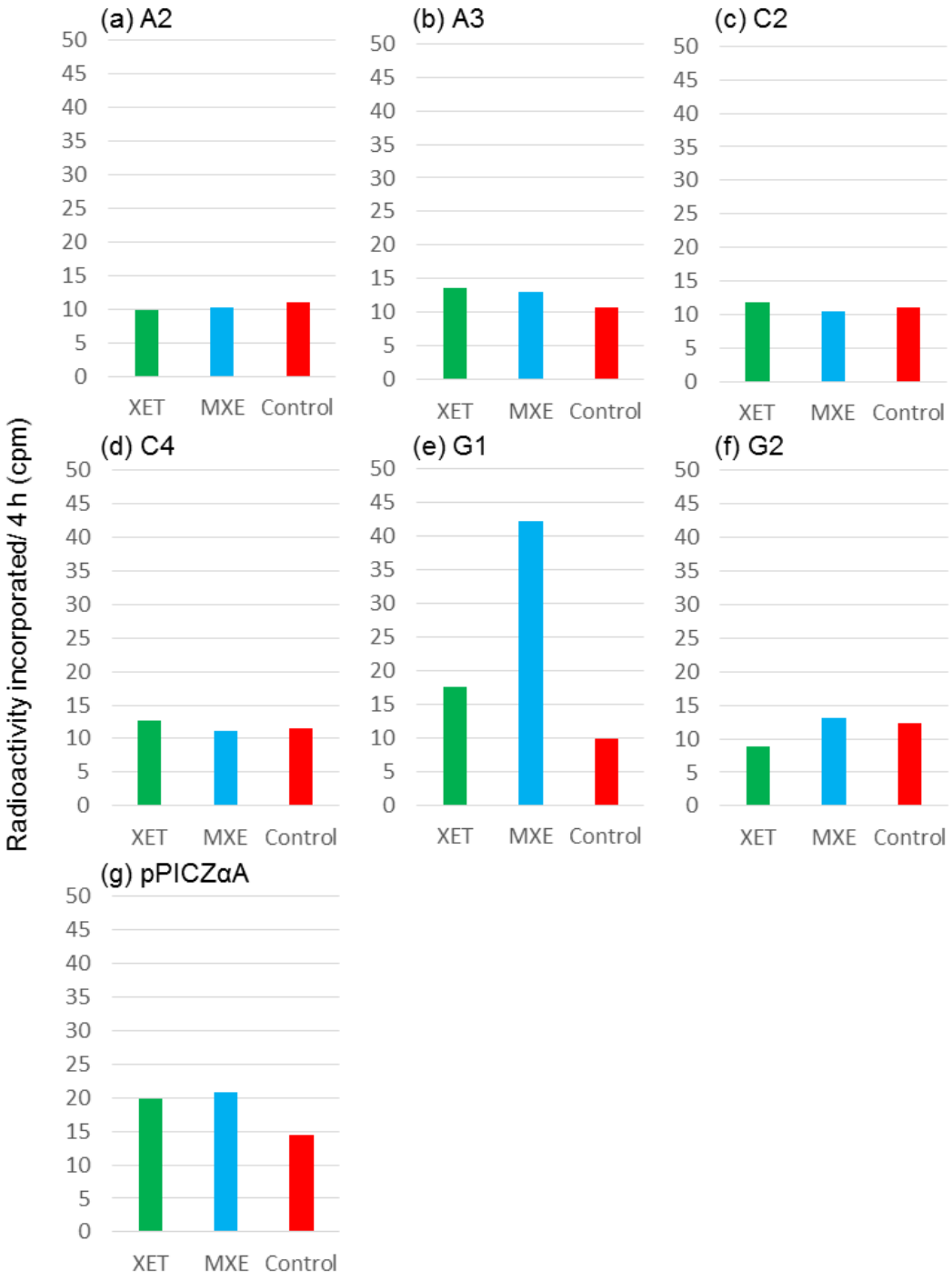


Figure 44: Preliminary assays for XET and MXE activity of candidate MXE genes; A, C and G.
Pichia-secreted products from different colonies of A, C and G candidate genes and a pPICZαA insert-free control were assayed for XET and MXE activity for 4 h (a) A2 (b) A3 (c) C2 (d) C4 (e) G1 (f) G2 and (g) pPICZαA. The number following the gene number indicates the orginal colony from which the *Pichia* strain originated. For example, C2 is from colony 2 of *Pichia* containing the C-gene insert.

6.12 Optimisation of construct G expression through use of an alternative expression medium

To determine the optimum medium for expression of candidate MXE proteins a range of different media was tested. In all cases the medium used was as described in 2.8.3, but with LB (low NaCl) substituted for YIP. *P. pastoris* cells containing a pPICZαA vector with a candidate gene insert from the new transcriptome were unable to survive in the presence of YIP, possibly due to the high salt content. The best growth medium for proliferation of transgenic *Pichia* was also tested with both BMGY and LB (low NaCl) + glycerol (1% v/v) resulting in comparable optical densities indicating equal growth for both media (results not shown).

Expression media tested included MM, MMY, BMM, BMMY and also LB (low NaCl) + MeOH (1% v/v). Of the media tested, expression was highest with LB (low NaCl) + MeOH. Negligible XET or MXE activity was observed following expression in MM or BMM. Expression was significant in the presence of MMY and BMMY but markedly higher in LB + MeOH (Figure 45). This suggested that LB (low NaCl) was required for expression of the target protein as it was not expressed in MM and BMM (which lack LB). Increased expression correlated with increased LB content; LB + MeOH (90% LB v/v) > BMMY (70% LB v/v) > MMY (10% LB v/v).

To ensure that the *P. pastoris* expression system was functional under the conditions used and comparable to that used for previous constructs, *Pichia* expressing 383 and pPICZαA were grown in BMGY and expressed in LB + MeOH for 24 h. pPICZαA refers to an insert-free plasmid which acts as a control expressing only endogenous *Pichia* proteins. XET activity observed with 383 was high, indicating expression of active recombinant transglycanases was possible using this system.

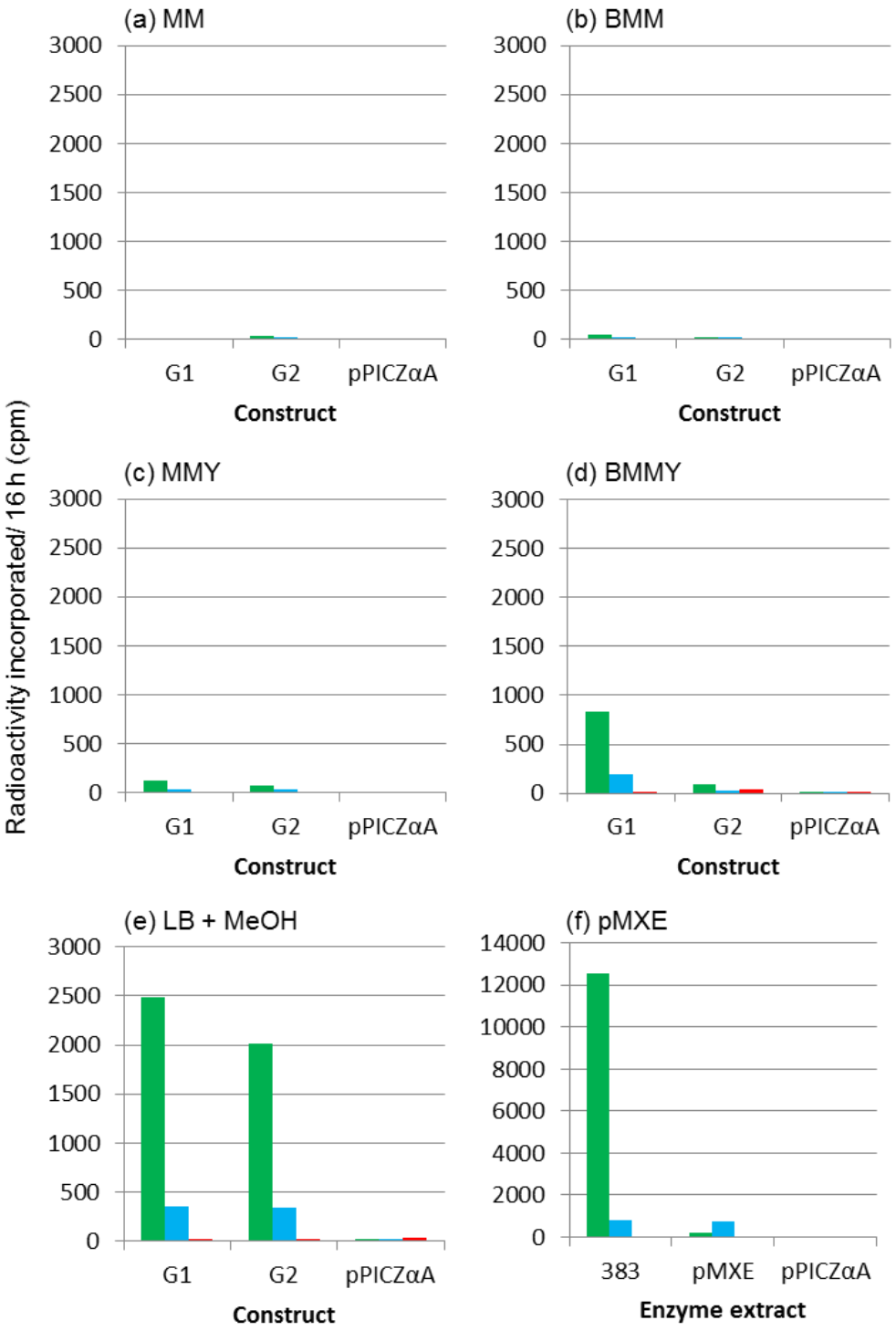


Figure 45: Optimisation of *Pichia* secretion of candidate G proteins
Different expression media – (a) MM (b) BMM (c) MMY (d) BMMY (e) LB (low NaCl) + MeOH (1% v/v) – were used under normal conditions. Secretion products were assayed for XET (**green**) and MXE (**blue**) activities, compared to a donor-free control (**red**) to determine the optimum medium for secretion of active candidate G proteins. To ensure the expression system was functional, construct 383 and a pPICZαA insert-free control were tested following expression in BMMY (f). XET and MXE activity of pMXE (f) was assayed to indicate the expected ratio of MXE: XET for the correct candidate MXE gene product.

6.13 An XET : MXE : CXE ratio comparable to that of pMXE is observed with the consensus candidate MXE

Following site-directed mutagenesis of candidate gene G, the consensus candidate MXE gene (Figure 46) obtained was inserted into the pPICZαA plasmid. The resulting vector was inserted into *P. pastoris* SMD1168H cells of which 14 colonies were precultured for a small scale expression assay. The highest expressing of these (colony 12) was used for a large scale expression assay.

```
383             MVSILSSSSSFCGLRRCLVLLVFLVQQAAAANFNQDFNITWAPDHVRVLDNSQLLQLTLD    60
A               ------------------------------GFYGDFQVEPVPDHV-IIQSDSLLQLTMD    60
C               ------------------------------GFYGDFQVEPVPDHV-IIQSDSLLQLTMD    60
G               ------------------------------GFYGDFQVEPVPDHV-IIQSDSLLQLTMD    60
HTG             ------------------------------GFYGDFQVEPVPDHV-IIQSDSLLQLTMD    60
                                              .*  **::  .**** :::...*****:*

383             QASGSGFVSKNQYLFGNIDMQIKLVPGNSAGTVIAYYLYSTASPNSHDELDFEFLGNVSG    120
A               KDSGGSVVSKSNYLFGYFNMKMKLISGNSAGTVITFYIFSDEA--NHDEIDFEFLGNYSG    120
C               KNSGGSVVSKSNYLFGYFNMKMKLISGNSAGTVITFYIFSDEA--NHDEIDFEFLGDYSG    120
G               KNSGGSVVSKSNYLFGYFNMKMKLISGNSAGTVITFYIFSDEA--NHDEIDFEFLGNYSG    120
HTG             KNSGGSVVSKSNYLFGYFNMKMKLISGNSAGTVITFYIFSDEA--NHDEIDFEFLGNYSG    120
                : **...***.:**** ::*::**:.********::*::*  :  .***:******: **

383             QPYILQTNVFTSGKGEREERINLWFDPTADFHTYSILWNPQIIIFSVDGTPIRVFPNNEK    180
A               DPYLLHTNIFASGVGNREQQFFLWFDPTADFHDYTIIWNPQQILFLVDGRAVRSFPNNE-    180
C               DPYLLHTNIFASGVGNREQQSFLWFDPTADFHDYTIIWNPQQILFLVDGRAVRSFPNNE-    180
G               DPYLLHTNIFASGVGNREQQFFLWFDPTADFHDYTIIWNPQQILFLVDGRAVRSFPNNE-    180
HTG             DPYLLHTNIFASGVGNREQQFFLWFDPTADFHDYTIIWNPQQILFLVDGRAVRSFPNNE-    180
                :**:*:**:*:** *:**:.  ********** *:*:**** *:* *** .:* *****

383             ALGVPYLNKQSMSLYSTLWNADGWATRGGLDKIDWTQAPFVASYTNFKADACTNYNTDYA    240
A               AIGVPYLKSQWMNVHLSLWNGETWATLGGLRRIDWNSAPFVASYSTFVGDSCFD-----S    240
C               AIGVPYLKSQWMNVHLSLWNGETWATLGGLRRIDWNSAPFVASYSTFVGDSCFD-----S    240
G               AIGVPYLKSQWMNVHLSLWNGETWATLGGLRRIDWNSAPFVASYSTFVGDSCFD-----S    240
HTG             AIGVPYLKSQWMNVHLSLWNGETWATLGGLRRIDWNSAPFVASYSTFVGDSCFD-----S    240
                *:*****:.* *.:: :***.: *** *** .***..*******:.* .*:* :     :

383             SAQACASSGDKWWNQPEYQTVASDDTSKLSWVRQNFLIYDYCSDIQRNPVP-PPECSNSH    300
A               ADSPCMAP--KWWNQAAYQSLSTSDAGSIQWVRENYLKYDYCYDIKLYPNGFPRECSNRG    300
C               ADSPCMAS--KWWNQAAYQSLSTSDASSIQWVRENYLKYDYCYDIKLYPNGFPRECSNRG    300
G               ADSPCTAS--KWWNQAAYQSLSTSDASSIQWVRENYLKYDYCYDIKLYPNGFPRECSNRG    300
HTG             ADSPCMAS--KWWNQAAYQSLSTSDASSIQWVRENYLKYDYCYDIKLYPNGFPRECSNRG    300
                : ..* :.  *****. **:::::.*:..:.***:*:* **** **:  *   * ****

383             I                                                               301
A               F                                                               301
C               F                                                               301
G               F                                                               301
HTG             F                                                               301
                :
```

Figure 46: ClustalW multiple sequence alignment by MUSCLE (3.8) of candidate MXE genes A, C, G and consensus (HTG), and 383
Multiple sequence alignment of new transcriptome candidate MXE genes expressed in *Pichia pastoris*. Construct 383 from the previous transcriptome, a putative XTH and member of the GH16 family of enzymes was included for sequence comparison, showing high conservation between all sequences.

Both unconcentrated (UC) and concentrated samples of colony 12 secretion products, hereafter referred to as heterotransglucanase (HTG), and a pPICZαA insert-free control were assayed for XET, MXE and CXE activity (Figure 47) alongside a putative recombinant XTH (concentrated 377) and pMXE. The aim of this was to determine whether the XET : MXE : CXE ratio observed for HTG was comparable to that of pMXE.

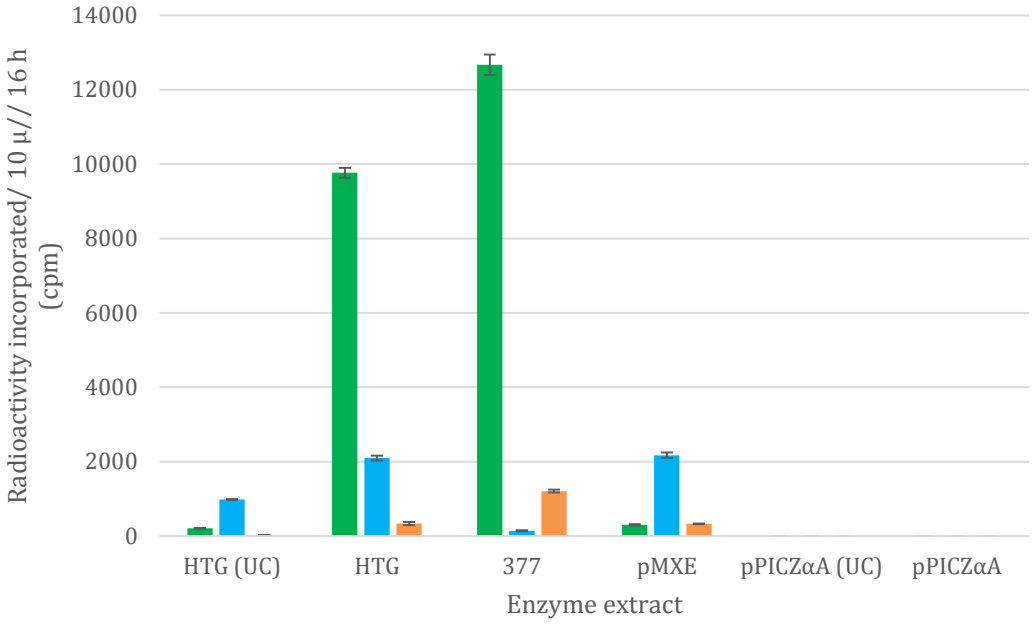


Figure 47: XET and MXE activity of pMXE and recombinant proteins
Under normal conditions, pMXE and *Pichia* expressed proteins were assayed in triplicate for XET (**green**), MXE (blue) and CXE (**orange**) activity pMXE and *Pichia* expressed proteins. All activities are cancelled for an enzyme-free control. This assay was conducted in triplicate and error bars indicate standard error.

Results showed a similar ratio of MXE : CXE activity for pMXE and concentrated HTG; 100 : 15.1 and 100 : 16.0 respectively. This ratio was significantly lower for unconcentrated HTG (100 : 1.9). However, unconcentrated HTG displayed lower activity for all transglucanase reactions and therefore the concentration of enzyme present within the extract may have been too low to produce detectable CXE activity. Interestingly, the ratio of XET : MXE activity dramatically increased following concentration of HTG, with XET increasing from 21.4% to 465% of MXE activity for unconcentrated and concentrated HTG respectively. Concentrating

the HTG extract would also have led to the endogenous *Pichia* proteins responsible for the degredation of MLG being concentrated too. Within a given reaction mixture the concentration of MLG molecules would remain constant between the concentrated and unconcentrated samples but the number of HTG proteins would have increased per MLG molecule in the concentrated sample, theoretically leading to increased MXE activity. However, the number of MLG degrading enzymes would also have increased per MLG molecule, increasing the possibility that both enzymes would employ the same MLG molecule. This could lead to a loss of detectable MXE product if the transglucanase product was degraded or the MLG was too small to act as a donor. pMXE meanwhile displayed XET activity of 14.1% of the MXE, consistent with that observed for unconcentrated HTG. This is consistent with the effect observed following concentration of constructs from the previous transcriptome, suggesting endogenous *Pichia* secretion products inhibit MXE activity.

Enzyme 377, a putative XET-active XTH, produced high levels of CXE product. However, this is a highly XET-active enzyme and maximum incorporation of radioactivity was achieved within the incubation time of this experiment. As a result, the ratio of XET : MXE : CXE cannot be meaningfully determined and despite its high incorporation of radioactivity, CXE activity could be a trace side reaction of an XET-predominant enzyme.

Unconcentrated HTG and pMXE showed comparable ratios of XET : MXE activity where MXE activity is far in excess of that observed for XET. However, due to the low amount of radioactivity incorporated by HTG (UC) and the detrimental effect of concentrating HTG on MXE activity, it became apparent that purification of HTG was pivitol for future work.

6.14 Timescale for XET, MXE and CXE activity for concentrated HTG

A 16 h timescale was established to study the XET, MXE and CXE activities of concentrated HTG, from which the initial rate of reaction for each activity could be estimated (Figure 48). Although unconcentrated HTG would have given a more accurate representation of MXE activity throughout the latter stages of the

timescale, under the conditions used here activity would have been undetectable at the lower timepoints as the solution was so dilute. Due to time constraints it was not possible to repeat this assay with a purified sample of HTG. Despite this, previous experiments regarding the initial rate of MXE activities indicated that it was not limited by degredation of MLG by the endogenous *Pichia* secretions and therefore we believed that an accurate ratio of XET : MXE : CXE activity for HTG could be determined as it does not plateu until later.

The XET : MXE : CXE ratio observed for concentrated HTG was 100 : 259 : 9.1 which was consistent with the pMXE ratio of 100 : 238 : 25 detected in section 6.5. This provides strong evidence that the recombinant HTG is a predominantly MXE-active enzyme.

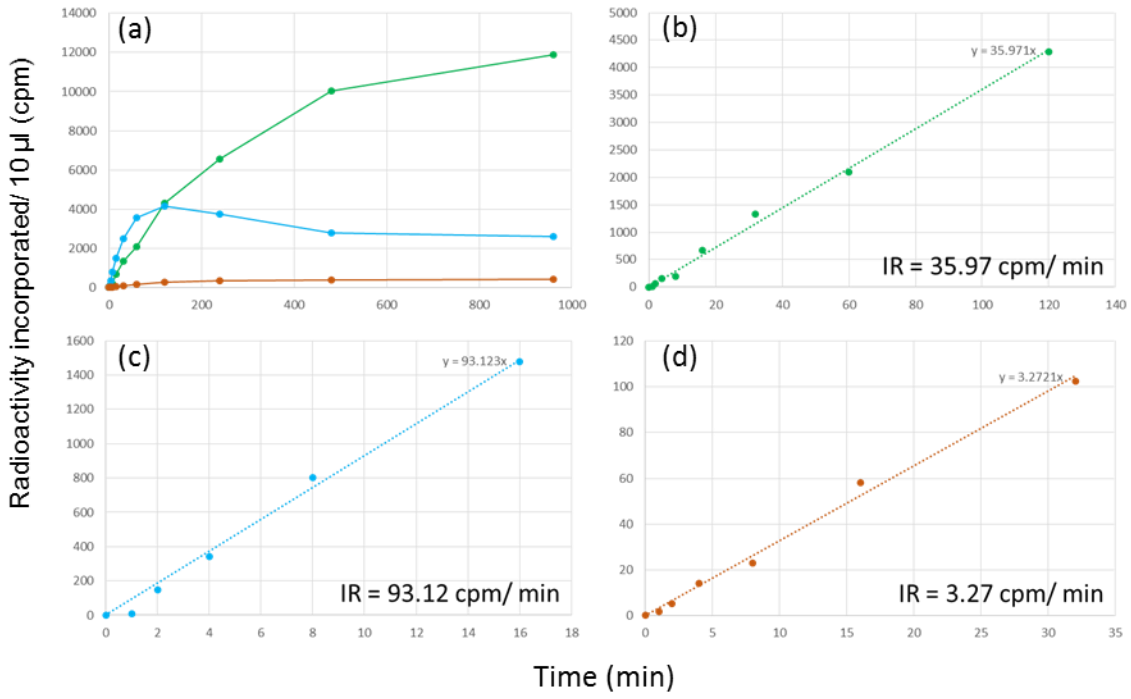


Figure 48: Timescale of XET, MXE and CXE activity for HTG (concentrated) (a) Full 16 h timescale of XET (**green**), MXE (**blue**) and CXE (**orange**) activities of concentrated HTG assayed for radioactivity incorporated per 10 µl enzyme extract. Also shown are the sections of linear initial activity for (b) XET (c) MXE and (d) CXE from which the initial rate of reaction was calculated. Results are averaged from duplicate samples. IR = initial rate.

6.15 Analysis of HTG acceptor substrate specificity

The secreted HTG protein of a high-expressing line of transformed *P. pastoris* was purified via the His-tag by IMAC (2.4.5) and assayed for XET and MXE activity with a range of different tritiated acceptors including XyG-, MLG- and cellulose-derived oligosaccharides. Due to limited availability of the radioactive acceptors, each donor–enzyme combination was incubated with 0.2 kBq of the appropriate acceptor substrate as opposed to the standard 1 kBq. Longer incubation times were employed for the detection of transglucanase activity.

The minimum structural requirement for XTHs has been reported to be XXG, of which the X at sub-site +1 is an absolute requirement (Lorences & Fry, 1993; Fanutti *et al*., 1993, 1996; Saura-Valls *et al*., 2008). HTG meanwhile did not appear to have this requirement for MXE activity as it was able to use [^{3}H]GXXGol as an acceptor substrate. In fact, under the conditions used, purified HTG (pHTG) MXE had the highest activity with this acceptor during a 20 h incubation, whilst XET activity was negligible. Due to a limited amount of [^{3}H]GXXGol, we were only able to study this reaction after 20 h incubation. It is therefore not possible to determine whether this lack of XET, which is a side reaction of HTG, was undetectable due to (a) a lack of specificity for the acceptor or (b) if activity was too low at this timepoint to be considered significant (Figure 49f). If the latter, longer incubation of the transglucanase reaction would have been sufficient to detect any significant XET activity. It is important to consider that the XET activity observed with various acceptor substrates in this assay is not a consistent proportion of the MXE activity. This could indicate that the MXE and XET activities for HTG have different acceptor substrate specificities.

Some MXE and XET activity was observed with a [^{3}H]GGXXXGol acceptor (Figure 49a). However, both activities were dramatically decreased during 40 h incubation. This assay needs to be repeated to determine whether this is a true representation of activity, possibly due to degradation of the acceptor due to the long incubation time, or it it is a result of experimental error. If this were a true activity it would indicate that, whilst not a requirement, an X at subsite +2 could result in higher MXE-activity.

High MXE activity was also observed with a [^{3}H]XXXGol acceptor (Figure 49b), especially after 40 h incubation where the difference between XET and MXE activity is proportional to that observed following a 20 h incubation with [^{3}H]GXXGol. This affinity of HTG MXE for [^{3}H]XXXGol is perhaps unsurprising as as previous studies have shown *Equisetum* MXE to have a significantly higher specificity for XXXGol over any other XGOs tested, such as XLLGol and XXGol (Fry *et al*., 2008a).

No significant homotransglucosylation with an MLG-oligo acceptor ([^{3}H]MLGO$_{7-8}$-ol (lichenase derived) or [^{3}H]MLGO$_8$-ol (cellulase derived) was observed (Figure 49c and Figure 49d respectively). In addition, HTG showed no specificity for heterotransglucosylation with [^{3}H]Cell$_6$ol (Figure 49e). The amount of radioactive acceptor used in this assay was low and therefore trace side reactions of HTG could not be distinguished. As a result, homotransglucanase and non-XGO heterotransglucanase activites could still occur as side reactions at low levels but they are not predominant activities of HTG.

Acceptor specificity results of HTG indicated a preference for X at subsite 2 as significantly higher MXE activity was observed with [^{3}H]GXXGol and [^{3}H]XXXGol. Determination of whether this X is a required or preferred residue for MXE activity requires the repeat of an acceptor specificity assay with the [^{3}H]GGXXXGol acceptor. Unlike XETs, HTG does not show a requirement for X at sub-site +1 as it showed high specificity for [^{3}H]GXXGol. However, all acceptors shown to produce significant MXE activity have an X at sub-site +3, suggesting that this could be an important requirement for HTG.

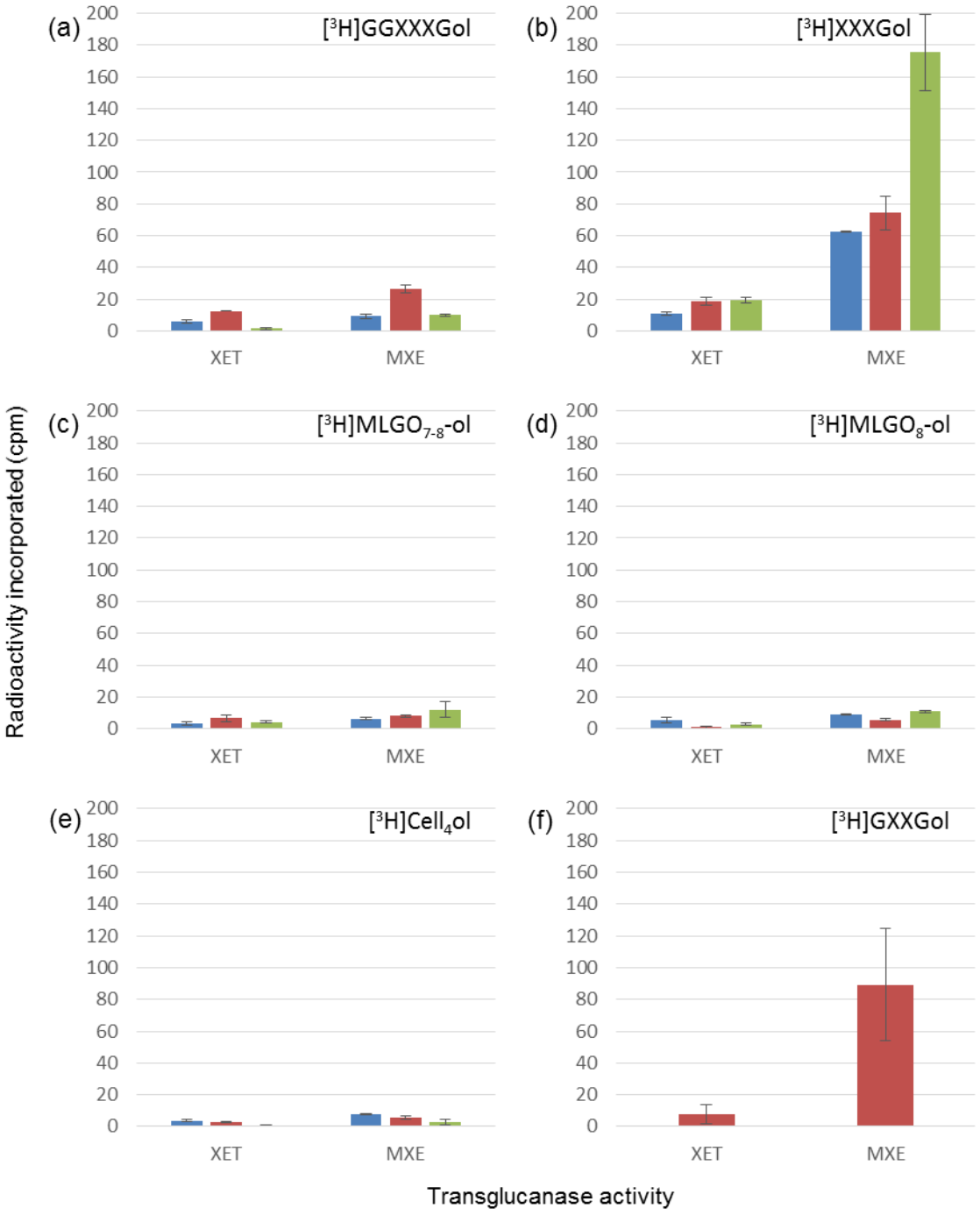


Figure 49: Acceptor substrate specificity of HTG for XET and MXE activity assays
Radioactivity incorporated in XET and MXE assays using 0.2 kBq of a range of different acceptors: (a) [^{3}H]GGXXXGol (b) [^{3}H]XXXGol (c) [^{3}H]MLGO$_{7\text{-}8}$-ol (d) [^{3}H]MLGO$_8$-ol (e) [^{3}H]Cell$_4$ol (f) [^{3}H]GXXGol incubated for 10 h (**blue**), 20 h (**red**) or 40 h (**green**). All results are cancelled for a donor-free control. Error bar indicates standard error for assays conducted in triplicate.

6.16 An SDS-PAGE gel could not confirm the purity of IMAC-purified HTG

Two SDS-PAGE gels (2.4.1) were run, one stained with Coomassie blue (2.5.4) and the other with silver nitrate (2.5.3), to allow determination of the purity of the IMAC-purified HTG. However, analysis was not possible as the bands failed to resolve due to overloading of unpurified HTG. Due to time constraints it was not possible to repeat the assay which had also aimed to allow us to semi-quantify the concentration of HTG by running it alongside a 10-fold dilution series of BSA (from 10 mg ml^{-1} to 1 µg ml^{-1}). We were unable to quantify HTG concentration via a Bradford assay (2.5.5) either as we could not ascertain whether the HTG was sufficiently pure and as such we have been unable to calculate the specific activity of the enzyme. However, XET: MXE: CXE ratios of HTG that correlate with those observed for pMXE, as well as acceptor substrate specificities which are unique from XET's, supports the proposal that the HTG gene encodes an enzyme responsible for MXE activity in *Equisetum*.

7 Discussion

7.1 Broad-spectrum assays identified a number of potential novel tranglycanase activities *in vitro*

Since the discovery of XET activity (Fry *et al*., 1992), a transglucanase implicated in the remodelling of the PCW during cellular expansion via reversible cleavage of xyloglucan tethers between cellulose microfibrils and subsequent yielding of the PCW to turgor pressure, there has been much interest in the discovery of other cell wall-remodelling enzymes. The discovery of MXE, MET and trans-β-xylanase (Fry *et al*., 2008a; Schröder *et al*., 2004; Franková & Fry, 2011; Ratke, 2014) highlighted the potential for other enzymes and/ or enzyme activities which catalyse the hetero- or homotransglycosylation of PCW polysaccharides. Transglycanases are hypothesised to be involved in cell wall remodelling and may impart certain characteristics, such as enhanced tolerance to drought and salt stress (Choi *et al*,. 2011) on the PCW which could be exploited for the nmengineering of more viable and advantageous crops.

7.1.1 Caveats of the qualitative and quantitative approaches for investigating novel transglycanase activities used in this work

Qualitative analysis for the identification of novel transglycanase activity was conducted on enzyme extracts sourced from beansprout, cauliflower, *E. arvense*, *E. fluviatile* and *H. lanatus*. I incubated these extracts with an SR-labelled oligosaccharide acceptor (Xyl_6-SR, XXXG-SR or Man_6-SR) and non-fluorescent potential donor polysaccharide. Transglycanase activity was associated with an increase in the molecular mass (M_r) of the fluorescent moiety as a portion of the donor polysaccharide is cleaved and grafted onto the NRT of the acceptor oligosaccharide, which is tagged at the RT with an SR moiety (Smith & Fry, 1991; Fanutti *et al*., 1993). As a result, transglycanase products remained at the origin of the silica-gel TLC plate and could be observed via short-wavelength (254 nm) fluorescence. However, fluorescence assays produced an unexpectedly broad range of positive results for different donor and acceptor combinations

suggesting some may have instead been artifacts of this method. It has previously been suggested that the non-polar SR moiety has a propensity to interact with a non-polar region within an enzyme, potentially XET present in the crude extract, resulting in its accommodation in the active site but with no transglycosylation (Ait-Mohand & Farkaš, 2006). This complex would also be detected as fluorescence at the origin due to its large M_r thus producing a false positive. Differentiation between trace novel transglycanase activities and potential artifacts is not always possible by this method alone.

To quantify potential transglycanase activity I conducted a radio-assay, as described by Fry *et al.* (1992), involving the incubation of an enzyme extract with a tritiated-oligosaccharide acceptor and potential donor polysaccharide. This method is more sensitive than the fluorescent assay previously described but it too has caveats. The detection of transglycanase activity *in vitro* does not confirm the presence of the same activity *in vivo*. The PCW is a complex domain and it is difficult to effectively mimic the *in vivo* conditions *in vitro* including, but not limited to, the proximity and availability of substrates, water availability, and the presence of inhibitors and/ or co-factors (Schröder *et al* 2004, 2009; Fry *et al.*, 2005). As such, a novel transglycanase activity detected *in vitro* needs further analysis to determine if it has physiological significance *in vivo*. Despite this, the assays outlined in this work are an effective initial strategy for detecting potential novel transglycanase activities which are worthwhile for further study.

7.1.2 Investigation into the potential for transglycanase activities incorporating pectic polysaccharides

Although transglycanase activities involving the main hemicellulosic polysaccharides of the PCW – XyG, MLG, mannans and xylans – have been identified, there are currently no known transglycanases that target pectic polysaccharides (Garcío-Romera & Fry, 1994). Since the discovery of xyloglucan–pectin linkages that are covalent in nature (Thompson & Fry, 2000), a number of studies have hypothesised that a cell-wall remodelling enzyme, such as a transglycanase, may be responsible for their formation (Keegstra *et al*., 1973;

Abdel-Massih *et al*., 2003; Cumming *et al*., 2005; Popper & Fry, 2008). Not only is there the potential for transglycanase activities which target backbone pectin residues but also in those that employ characteristic pectic side-chain moieties (Schröder *et al*., 2004).

7.1.2.1 No detection of xyloglucan : arabinan/galactan transglycanase activity speculated to create xyloglucan–pectin crosslinks

The formation of xyloglucan–pectic linkages, as described in the literature, would require a transglycanase capable of employing a pectic polysaccharide (or associated side-chain, e.g. β-(1,4)-galactan or α-(1,5)-arabinan) as the acceptor and xyloglucan as the donor, potentially leading to the formation of a complex between the three main polysaccharide domains of the PCW: cellulose, hemicelluloses and pectin. Popper & Fry (2008) tested this hypothesis using an α-(1,5)-arabinan or β-(1,4)-galactan acceptor and xyloglucan donor in the presence of crude enzyme extracts and found no formation of a xyloglucan–pectin complex. This is consistent with our observations following incubation of an [^{3}H]Ara$_8$-ol or [^{3}H]Gal$_6$-ol acceptor and tamarind XyG donor with a crude beansprout extract. Therefore, I have been unable to detect a transglycanase capable of creating xyloglucan–pectin linkages.

7.1.2.2 Apparent AXE and GXE activity was due to XyG contamination of commercial donor polysaccharides rather than a novel transglycanase

A central component of this work resulted from the detection of potential α-arabinan : xyloglucan and β-galactan : xyloglucan transglycanase activities in beansprout during broad-spectrum fluorescence- and radio-assays. However, further analysis confirmed that activity detected was representative of XET activity, rather than that of a novel transglycanase, due to contamination of donor polysaccharides with trace quantities of XyG. Furthermore, although we have been unable to conclusively prove that a transglycanase activity involving the grafting of a donor α-arabinan or β-galactan polysaccharide onto a XGO acceptor

does not exist, it is difficult to speculate a role for this activity *in vivo* as little is known about the function of RG-I side-chains in the PCW.

7.1.2.2.1 XET activity is detectable at extremely low concentrations of XyG

It was interesting to discover that some XTHs are able to show detectable XET activity even at extremely low XyG concentrations; we observed significant XET activity with a XyG concentration as low as 12.2 pg μl^{-1}. Frequently in the literature, the K_M of known XTHs is elucidated but this is possibly the first assay which has specifically aimed to determine the minimum concentration at which XET activity is detectable. This result has implications for future work, especially that aiming to identify novel heterotransglycanase activities with an XGO acceptor, as even trace contaminants of the potential donor polysaccharide by XyG can produce false positives.

7.1.2.3 The possibility of other xyloglucan : pectic polysaccharide transglycanases

A pattern has emerged indicating that transglycanases reside within the same GH group as their hydrolase counter-parts. It has been determined that XTHs responsible for both XET and XEH activities reside in family GH16 (Johansson *et al*., 2004). The same is true of lichenases (MLG hydrolases) and the MXE-active HTG, both in GH16 (Strohmeir *et al*, 2004; see 6.13), and MET and endo-β-mannanases which are members of GH5 (Bourgault *et al*., 2005). Importantly, all known transglycanases are retaining rather than inverting enzymes.

In my broad-spectum assay, we observed significant transglycanase activity with both pectin and RG-I as potential donor polysaccharides following incubation with a [^{3}H]XXXGol acceptor. However, it is hard to imagine a known hydrolase capable of carrying out this transglycanase activity as the most likely candidates, rhamnogalacturonases (Schols *et al*, 1990; Petersen *et al.*, 1997) and endopolygalacturonases (Biely *et al*, 1996), are inverting enzymes. In addition, it has been proposed that there is no requirement for transglycanase activity for the incorporation of pectins with little or no branching into the PCW (Schröder *et*

al., 2009). The ease with which these pectins are generally extracted from the PCW suggests they are only bound by either Ca^{2+} or weak covalent bonds, rather than glycosidic bonds characteristic of transglycanases.

It has been hypothesised that links between xyloglucan and pectic polysaccharides could occur via the side chains of RG-I (Thompson & Fry, 2000; Schröder *et al.*, 2009): α-(1,5)-arabinan, β-(1,4)-galactan and/ or type 2 arabinogalactan. The most likely candidate hydrolases for exhibiting xyloglucan : arabinan transglycanase activity are *endo*-arabinanases (EC 3.2.1.99; GH 43) but they are inverting enzymes. As a result, it is unlikely that *endo*-arabinanases would be able to catalyse transglycanase activity. With respect to a potential galactan : xyloglucan transglycanase activity, there are retaining *endo*-galactanases in GH16 and GH30 but they hydrolyse β-(1,3)- (EC 3.2.1.181) and β-(1,6)-galactans (EC 3.2.1.164) respectively, which are not abundant in pectin. Interestingly, there is an *endo*-β-(1,4)-galactanase (EC 3.2.1.89) in GH 53 which hydrolyses β-(1,4)-D-galactosidic linkages in plant arabinogalactans. We identified significant transglycanase activity with an arabinogalactan (AG Sigma) donor following incubation with beansprout, cauliflower and tomato extracts. This indicates a potential novel heterotransglycanase activity which warrants further investigation.

7.1.3 Other potential novel heterotransglycanase activities have been detected but further analysis is required

Numerous potential transglycanase activities were implicated in my broad-spectrum radio-assay. Reaction mixtures were incubated for 16 h to allow trace transglycanase activities to be observed. However, as maximum incorporation of radioactivity had been reached with XET activity by 16 h, it is not possible to quantify other potential transglycanase activities as a percentage of the XET activity observed. As shown in my work regarding the potential AXE/ GXE activity detected in beansprout, it is possible that any potential transglycanase activity detected could be the result of contamination of the potential donor polysaccharide with trace quantities of XyG. XET activity has been recorded with

XyG concentrations as low as 12.2 pg μl^{-1}. Although it would theoretically be possible to remove such minute traces of XyG from potential donor polysaccharides with XEG it would be near impossible to confirm its removal. However, it would be possible to detect genuine novel transglycanase activities by determining whether the ratio of XET activity to a specific potential novel transglycanase activity differs between different enzyme extracts. For this to be possible, a timescale of each transglycanase activity for each enzyme extract would have to be conducted thus allowing comparison of initial rates of reaction. If the proportion of XET : novel transglycanase activity for a specific donor polysaccharide remained consistent between enzyme extracts then it could be assumed, as the XyG contamination would be the same throughout, that the activity observed is representative of XET activity. Meanwhile, if the relative proportion of these two activities differed between enzyme extracts this would strongly indicate a possible novel transglycanase activity.

Detection of a novel transglycanase activity does not necessarily indicate the presence of a novel enzyme. It is possible that some transglycanase activities observed in our experiment may occur as a side-reaction of known transglycanases such as XET-active XTHs. However, it is difficult to envisage how known transglycanases could accommodate some of the potential donor polysaccharides tested due to active site specificities. For example, it is improbable that XET-active XTHs, which require a minimum donor polysaccharide mofit of β-(1,4)-linked GG (Fry *et al*., 1992; Fanutti *et al*., 1993, 1996; Saura-Valls *et al*., 2008), could catalyse a reaction with a pectin or β-(1,3)-glucan.

Of particular interest are transglycanase activities we detected that are consistent with those proposed in the literature. For example, we identified significant activity with a glucuronoxylan donor and [^{3}H]XXXGol acceptor in *E. arvense, E. fluviatile*, *H. lanatus* and snowdrop extracts. A previous study by Kosík *et al*. (2010) observed potential enzyme activity for glucuronoxylan : xyloglucan-SR and concluded it was likely to be artificial. However, there is no proof of this. We also detected high levels of transglucanase activity in all enzyme extracts with

a HEC donor. Structurally HEC is similar to xyloglucan and has previously been shown to be a suitable substrate for some XET-active XTHs; Hrmová *et al*. (2007) reported activity which was 44% of that observed for XyG. Without further analysis I am unable to quantify transglycanase activity with a HEC donor as a proportion of the observed XET activity (as it is limited by substrate availability). However, by comparing the activity observed following maximum incorporation of radioactivity (~10 000 cpm) by XET and the activity observed with a HEC donor, we could identify enzyme extracts where activity is less than the 44% of XET activity observed by Hrmová *et al*. (2007). The differences in activity with HEC proportional to XET activity between species is a good indicator of novel transglycanase activity. Although we have been unable to confirm the presence of any novel transglycanase activites we have detected numerous possible candidates.

7.1.4 Future work

Due to time restrictions we were unable to conduct more than a preliminary broad-spectrum novel transglycanase assay but the scope for potential new work is huge. Of greatest importance, given the work already completed, is the establishment of a timescale allowing calculation of the initial rate for each activity using all current potential donor polysaccharides and enzyme extracts with a [^{3}H]XXXGol acceptor. Comparison of initial rates will allow expression of a potential novel transglycanase activity as a proportion of the XET activity observed. If this potential novel heterotransglycanase activity, using a XGO acceptor, were to differ between enzyme extracts for the same donor polysaccharide, it would be a strong indicator of significant novel heterotransglycanase activity rather than contamination of the donor with XyG.

Another extension to this experiment would be to increase the range of acceptor oligosaccharides used, allowing the observation of homo-transglycanase activities as well as untested hetero-transglycanase activities. The number of possible different donor-acceptor-enzyme extract combinations is vast, further

highlighting the potential for as yet undiscovered novel transglycanase activities within the PCW.

7.2 Successful expression of five recombinant XTHs and a putative MXE from *Equisetum*

7.2.1 All 5 recombinant GH16 XTHs displayed high XET activity with MXE activity as a side-reaction

All recombinant GH16s were highly XET-active XTHs and displayed extensive homology in their primary structures yet their specificity for XET activity differed. The translation product of construct 377, which was the most XET-active XTH tested, displayed the lowest MXE activity: 0.17% of the XET activity. This is consistent with the observations of Hrmová *et al*. (2007) and Fry *et al*. (2008a) who detected MXE activity which was ~0.2% of the XET activity of Poalean enzymes. Recombinant protein 383, which is also a highly XET-active XTH, produced a ~19-fold higher proportion of MXE activity – 3.76% of XET activity – than with 377. It can therefore be concluded that construct 377 is a more specific XET than 383 due to a lower incidence of side-reactions.

It is perhaps unsurprising, in view of the relaxed donor substrate specificity of XET-active XTHs, that they can also catalyse MXE activity. The minimum requirement for an XET donor polysaccharide is a GG moiety (Fry *et al*., 1992; Fanutti *et al*., 1993, 1996; Saura-Valls *et al*., 2008). As MLG can be used as a donor polysaccharide, it is also plausible that cellulose with its β-(1,4)-linked glucose backbone could also be used as a donor polysaccharide by XET-active XTHs. This is supported by the low level of CXE activity observed with all GH16 XTHs as well as HTG. However, it is speculative as to whether CXE is a physiologically significant activity *in vivo* or whether it is a side-reaction observed *in vitro*.

7.2.2 The recombinant HTG has MXE activity correlating with that observed from *Equisetum* pMXE

Through use of a heterologous *P. pastoris* expression system, we have successfully identified an *E. fluviatile* gene which encodes HTG, a putative MXE-active protein. Perhaps unsurprisingly, this enzyme is an XTH homologue and member of the GH16 family, as had previously been proposed by Fry *et al*.

(2008a). The ratio of XET : MXE activity observed with the recombinantly expressed HTG was comparable to that of the highly MXE-active pMXE extract from *Equiseum*, both producing a ratio of ~ 1 : 2.5. It is exciting that this is the first plant enzyme which has conclusively shown MXE activity in excess of XET and that predominatly catalyses a hetero-transglycanase reaction.

Interestingly, the structural requirements for acceptor oligosaccharides between XET-active XTHs and this MXE-active HTG are significantly different. Putative XET-active XTHs require a minimum acceptor oligosaccharide of XXG, with the X at sub-site +1 being an absolute requirement and the X at sub-site +2 being advantageous for transition state stabilisation (Lorences & Fry, 1993; Fanutti *et al*., 1993, 1996; Saura-Valls *et al*., 2008). However, in acceptor specificity assays, HTG showed highest transglucanase activity in the presence of [^{3}H]GXXGol as acceptor, followed by [^{3}H]XXXGol as the second most active acceptor. This indicates that, unlike XTHs, HTG does not have a requirement for X at sub-site +1. Also, although HTG preferred acceptor substrates with an X at +2 it is not essential as very low but significant activity was observed with the acceptor [^{3}H]GGXXXGol. No homo-transglucanase activity was observed with either MLG + [^{3}H]MLGO or hetero-transglucanase activity with MLG + [^{3}H]Cell$_6$ol. In this study, MXE activity was only detected with acceptors that had an X at sub-site +3, although a XGO acceptor without X at +3 was not tested. However, this could potentially indicate that a X residue at +3 could be involved in stabilisation of the glycosyl-enzyme intermediate formed during MXE-activity of HTG but further analysis is required to confirm this.

Our observation of different acceptor sub-site specificities between XET-active XTHs and the MXE-active HTG is another characteristic which distinguishes HTG from conventional XTHs. This along with the speculated role for MXE in cessation of cell growth compared to XET's proposed role in promoting cell elongation further support the hypothesis that these two activities are catalysed by different proteins, even if they do have a high degree of primary sequence homology.

7.2.3 Glycosylation is an important post-translational modification for the carrying out of XET and MXE activities

It has been proposed that *N*-glycosylation of conserved residues 5-15 amino acids downstream from the active site of XTHs is vital for XET activity (Campbell and Braam, 1999a; Henriksson *et al*., 2003). However, the retention of significant XET activity by deglycosylated *Ptt*XET16A indicates this is not an essential requirement for all XTHs (Johansson *et al*., 2004). Deglycosylation of recombinant GH16 proteins 398 and 383 resulted in a significant decrease in XET activity for both enzymes. However, the effect of deglycosylation was more pronounced with 398, a highly specific XET-active XTH, suggesting that it may be integral for proper XET function. Deglycosylation of pMXE decreased MXE-activity to around that of the background indicating a requirement of MXEs for glycosylation. The effect of dephosphorylation effect on XET was inconsistent due to the unreliability of results for 398 and 383. However, results for pMXE indicated a two-step decrease in activity which could potentially indicate two main phosphorylation sites important for full activity of the enzyme.

7.2.4 Recombinant GH17 and GH64 proteins produced no detectable XET activity but may still be viable transglycanases

No transglucanase activity was observed with any GH17 or GH64 recombinant proteins in the presence of an XGO acceptor. However, this does not rule out the possibility that they could catalyse transglycanase activity with other donor polysaccharides and acceptor oligosaccahrides, especially as they have homology to other known XTHs. It is perhaps unsurprising that GH17 enzymes did not have XET, MXE or CXE activity as all enzymes known within this family catalyse reactions using β-(1,3)-glucans. There has already been a β-(1,3)-glucan transglycanase (EC 2.4.1.-) detected in this family from bacteria and thus it would be worthwhile to test our recombinant GH17 proteins for β-(1,3)-glucan homo-transglucanase activity.

By contrast, we proposed that transglycanase activity is less plausible for GH64 proteins as all activities detected in this family are inverting hydrolases whereas

all known transglycanases are retaining. The homology observed between GH17 and GH64 is probably indicative of a common enzyme class of β-(1,3)-glucanases (EC 3.2.1.39) rather than an indicator of potential transglycanase activity.

7.2.5 Future work

In order to fully characterise the HTG protein identified in this work, further investigation is required such as studies of K_M, V_{max} and analysis of donor and substrate specificities with a more extensive range of poly- or oligosaccharides. Of particular importance is determination of the specific activity, and thus K_{cat} of HTG. It would also be interesting to identify residues and/ or post-translational modifications, such as *N*-glycosylation and phosphorylation that distinguish it from XET-active XTHs.

A useful initial step in the prediction of the crystal-structure HTG would be 3D-modelling of the protein based upon a lichenase and/ or *Ptt*XET16A template to allow visualisation of potential aromatic residues within the active site of the enzyme that could influence donor and acceptor sub-site preferences. It would be interesting to determine whether the loop extension between β8 and β9 in XEH-active XTHs, which distinguishes it from XET-active XTHs, is present is lichenases but absent in HTG thus indicating a structural component that confers hydrolase activity rather than transglycanase activity.

Now that we have identified the gene which encodes an MXE-active HTG it is possible to investigate what the function of MXE activity is in plants through the use of knock-out mutants. It might also be interesting in the future to express the gene in plants, such as cereals, that have MLG but do not have MXE to see the effect this has on the plants' phenotypes. Although this may not be possible due to limitations resulting from the differences between *Equisetum* and other plant cell walls, it could indicate whether the genetic engineering of crop plants to express this gene is feasible.

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